Polyandry and Postcopulatory Sexual Selection in Yellow Dung Flies

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Marco Demont

aus

Vella GR

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Prof. Dr. Wolf U. Blanckenhorn

(Vorsitz, Leiter der Dissertation)

Dr. Luc F. Bussière

Prof. Dr. Tony Wilson

Prof. Dr. Paul I. Ward

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General Introduction

Sexual selection

Sexual selection arises because individuals vary in reproductive success (Andersson 1994; Darwin 1871). It is frequently a very powerful evolutionary force, because variation in reproductive success often exceeds that of other fitness components, including survivorship (Arnqvist & Rowe 2005; Hoekstra et al. 2001; Kingsolver et al. 2001). Over the past decades, many studies have documented the power of sexual selection in shaping and diversifying morphological, physiological, and behavioral traits of both sexes (Andersson 1994; Arnqvist & Rowe 2005; Birkhead et al. 2009; Birkhead & Moller 1998; Simmons 2001).
Sexual selection is dominated by two main processes: competition within one sex (intrasexual selection), usually males, for access to members of the other sex, usually females; and mate choice exerted by the sex in short supply (intersexual selection) (Andersson 1994; Darwin 1871). The conventional sex roles are characterized by male competition and strong female choice (and not vice versa) because the potential reproductive rate of males is usually higher than that of females (Bateman 1948; Clutton-Brock & Vincent 1991; Trivers 1972; Williams 1966). The important role of intrasexual selection for trait evolution is relatively well recognized because it is easy to observe and understand (Andersson 1994). In contrast, the ultimate causes and consequences of female preferences remain controversial (Andersson 1994; Arnqvist & Rowe 2005).

Sexual selection extends beyond precopulatory processes, as securing mates is often not sufficient to determine reproductive success (Eberhard 1996; Parker 1970c; Simmons 2001). As for precopulatory sexual selection, two postcopulatory processes can be recognized: male ejaculates compete for fertilization (sperm competition) and females may exert a preference for the sperm of certain males (cryptic female choice). Sperm competition is indisputably an important evolutionary force (Parker 1970c), and has driven the evolution of many male traits involved in the avoidance or engagement in competition for the fertilization of a set of ova (Birkhead et al. 2009; Birkhead & Moller 1998; Simmons 2001). Additionally, sperm competition has important evolutionary implications far beyond the context of insemination and fertilization success, including life history evolution and speciation (Hosken 2001; Pitnick & Miller 2000; Simmons 2001). In contrast, the role of females in determining fertilization

outcomes and driving evolution has until recently received less attention. This imbalance is glaring, because females provide the selective environment in which postcopulatory sexual selection occurs, and they are certainly not passive agents in the process (Lloyd 1979; Parker 1970c). Thornhill (1983; 1984) introduced the term cryptic female choice to describe female processes occurring during or after copulation that bias paternity towards a certain male. In subsequent research, over 20 potential mechanisms enabling females to exert cryptic choice have been described (Eberhard 1996). Fifteen of these mechanisms seemed applicable to insects, categorized into the following five subgroups: female influences on remating, sperm transfer, sperm storage, sperm utilization at the time of fertilization (i.e. sperm selection), and differential investment in offspring (Eberhard 1996; Eberhard 1997; Simmons 2001).

Although any of these female influences could easily have a large impact on the fertilization success of a particular male, such influences are rarely thoroughly tested, and some of these mechanisms (e.g. sperm selection) have so little empirical support that their importance remains doubtful (Birkhead 1998; Birkhead 2000; Eberhard 2000; Kempenaers et al. 2000; Pitnick & Brown 2000; Simmons 2001). This paucity of evidence is partly explained by the fact that empirically examining cryptic female choice is very challenging, and some of the techniques that have been used suffer from practical limitations (Birkhead 2000; Bussiere et al. 2009; Hall et al. 2010). For example, quantifying sperm in storage using phenotypic markers such as sperm length is difficult, and complete unequivocal assignment is often impossible (Hellriegel & Bernasconi 2000; Otronen et al. 1997). Furthermore, sperm size may not be selectively neutral with respect to competitive ability (Gage 1994; Gage & Morrow 2003), which could easily obscure the relationship between insemination success, sperm storage and paternity. As a consequence, our knowledge of the mechanisms of sperm transfer, storage and utilization is limited, and data that directly link the number of stored sperm to paternity are largely missing (Simmons 2001). Hence, the relative contributions of male (sperm competition) and female (cryptic female choice) mechanisms to differential fertilization success is currently unknown (Snook 2005).

Polyandry

Polyandry (females mating with more than one male) is a prerequisite for postcopulatory sexual selection and very common in the animal kingdom. Nevertheless, the evolutionary causes and far-reaching consequences of polyandry remain the subject of debate (Arnqvist & Kirkpatrick 2005; Arnqvist & Nilsson 2000; Arnqvist & Rowe 2005; Evans & Simmons

2008; Garcia-Gonzalez & Simmons 2007; Jennions & Petrie 2000; Puurtinen et al. 2009; Simmons 2005). This is especially true if there are no obvious direct benefits associated with female remating, for example the replenishing of sperm stores or the acquisition of food from mating partners. In such cases, repeated mating by females might arise via a number of alternative nonadaptive (e.g. correlated response to sexual selection on multiple mating by males (Halliday & Arnold 1987)) or adaptive mechanisms, including the acquisition of high quality or compatible genes (indirect genetic benefits) (Jennions & Petrie 2000; Tregenza & Wedell 2000; Zeh & Zeh 2001). The relative importance of each of these alternatives is currently unknown both in general and for many specific examples of female polyandry.

Many important laboratory studies have attempted to clarify the forces acting on female remating rates (Ivy 2007; Martin & Hosken 2003; Tregenza & Wedell 2002; Zeh & Zeh 2006), but extrapolating results to the natural situation in wild populations is difficult in part because we seldom know if laboratory settings reflect realistic conditions in wild populations (Bretman & Tregenza 2005; Simmons et al. 2007). This lack of information constrains progress clarifying the causes of polyandry and its implications. Consequently, more documentation of natural levels of polyandry in wild populations are needed (ideally featuring analyses of its spatial and/or temporal variation), as are studies of the ecological and evolutionary factors that alter selection on wild female remating rates (Wilson 2009).
Assessing the degree of polyandry by directly observing mating in the field poses a challenge, especially for small and mobile species such as insects. One solution is to genotype the sperm within the sperm stores of females to assess the number of mates (Bretman & Tregenza 2005; Chapuisat 1998; Krieger & Keller 2000). Since copulations may not always result in successful sperm transfer and sperm from recent mates may have displaced sperm from previous males, this estimate (the genetic mating frequency) may be an underestimate of the actual mating frequency in the field (the social mating frequency) (Simmons et al. 2007). Nevertheless, the genetic mating frequency is a good measure of the *minimum* degree of polyandry prevalent in the wild, a parameter that is probably more important for male sexual behaviour than the social mating frequency of females.

Varying levels of polyandry do not only affect the *number of ejaculates* that compete within the female for fertilization of the ova, but also *how much ejaculate* from each male is present in the contest (Engqvist & Reinhold 2006; Gage et al. 1995; Galvani & Johnstone 1998; Parker et al. 1996; Parker et al. 1997; Wedell et al. 2002). Several studies have shown that

males adjust their reproductive behaviour according to the risk of sperm competition (indicated by the level of polyandry). For example, when subject to higher risks of sperm competition, elephant seals (*Mirounga angustirostris*) show more aggressive behaviour against rival males (Leboeuf & Peterson 1969), and flour beetles (*Tenebrio molitor*) increase their mate guarding (Gage & Baker 1991). Additionally, males allocate investment in sperm in response to sperm competition risk (Pizzari et al. 2003; Wedell et al. 2002). Comparative studies have consistently shown a positive relationship between the degree of polyandry (an index of sperm competition) and relative testis size (a standard index of investment in sperm) amongst related taxa (Gage 1994; Hosken 1997). Within species, males adjust their ejaculate expenditure during a particular mating event according to cues arising from other conspecifics (males and females). Several studies have provided evidence that the presence of rival males can result in increased ejaculate size (Gage 1991; Pound & Gage 2004). Males also strategically allocate their sperm according to female mating status and/or quality (Martin & Hosken 2002; Wedell 1998). Exactly how males detect female mating status (e.g. virgin vs. mated) and/or the number of sperm or ejaculates stored by females is often unclear (Engqvist 2007). Importantly, the relationship between sperm competition risk (the probability that a female will mate with more than one male) and sperm competition intensity (the number of males involved in sperm competition) is not always straightforward. For example, there may be few males present at mating sites (i.e. low sperm competition risk), but females might have already mated several times and stored sperm from several males (i.e. high sperm competition intensity). This example illustrates that cues arising from other males (e.g. operational sex ratio) and cues arising from the female (e.g. female mating status) may affect males very differently. Just as for research on polyandry in general, empirical research on strategic sperm allocation (a consequence of varying levels of polyandry) is predominantly based on laboratory studies. Data from wild populations that directly assess the number of males involved in sperm competition are needed to help test predictions derived from theoretical models on the evolution of male sperm expenditure.

The yellow dung fly

Yellow dung flies, *Scathophaga stercoraria*, are a model system for studying sexual selection since Parker's pioneering work in the 1970's (Parker 1970a; Parker 1970b; Parker 1970c; Parker 1970d; Parker 2001; Parker & Simmons 1994; Parker & Thompson 1980; Simmons 2001; Simmons et al. 1999; Ward 2007). Male interactions seem to drive precopulatory

sexual selection (Jann et al. 2000; Parker 1970a; Parker 1970b), but females retain some control over postcopulatory processes (Ward 2007). As in many of the Diptera, female yellow dung flies have multiple sperm storage organs (spermathecae) into which males cannot directly insert sperm (Hosken 1999; Hosken et al. 1999; Hosken & Ward 2000; Simmons et al. 1999). Instead, males ejaculate into the bursa copulatrix (Hosken 1999; Simmons et al. 1999), with the phallosome (endophallus) almost directly abutting the spermathecal duct openings (Hosken et al. 1999). Female yellow dung flies have three spermathecae (one called the singlet on one side of the body, and a pair collectively called the doublet on the opposite side), each with its own narrow duct (Hosken et al. 1999). Several lines of evidence suggest a possible role for these organs in sperm choice. First, theoretical work has shown that separate sperm stores could allow differential storage rates (e.g. transport to the spermathecae) and differential use for different males (Hellriegel & Ward 1998). This theoretical work is complemented by observations that the singlet and doublet spermathecae have independent musculature in live preparations (L. F. Bussière, unpublished observations), and thus could potentially assist in sorting sperm for subsequent sperm selection during oviposition (Hellriegel & Bernasconi 2000). In fact, females seem able to selectively choose the sperm from a particular male at the time of fertilization (i.e. adaptive sperm selection), and thereby match the genotypes of her offspring to environmental conditions (Ward 2000). However, even in this model species, the exact mechanisms underlying non-random paternity are far from clear, and more studies are needed to establish the conditions favouring cryptic choice, the mechanisms that mediate it, and its importance for male and female fitness.

Outline of the thesis

Three kinds of empirical studies are most promising for promoting our understanding of polyandry and postcopulatory sexual selection. First, testing models of female preference evolution in a quantitative genetic framework can reveal postcopulatory mechanisms in action (Chapter 1). Second, a detailed knowledge of the mechanisms of sperm transfer, storage, and utilization is essential to understand male and female influences on differential fertilization success, as is directly linking the number of stored sperm with paternity success (Chapter 2 and 3). Third, field data on sperm storage, paternity, and prevalent levels of polyandry are a necessary to validate previous laboratory experiments and may suggest new avenues for experimental research (Chapter 4 and 5).

Chapter 1 is an essay about a sexually selected sperm process in the dung beetle *Onthophagus taurus* that was initially described by Leigh Simmons and Janne Kotiaho (2007). Their quantitative genetic study investigated how sexual selection favoured the evolutionary divergence of sperm size. Chapter 1 considers whether postcopulatory sexual selection can shape sperm morphology in the same way that precopulatory female preferences affect the evolutionary divergence of male secondary sexual traits.

Chapter 2 describes the development and application of competitive microsatellite PCR for quantifying relative contributions of sperm in storage. It documents how DNA template characteristics affect PCR amplification and describes an application of the method to examine the influence of mating interval on patterns of sperm storage in twice-mated female yellow dung flies.

Chapter 3 applies the competitive microsatellite PCR method (described in Chapter 2) to relate biases in sperm storage to sperm use. We manipulate the occurrence and timing of oviposition relative to two matings in a controlled laboratory experiment using yellow dung flies. By genotyping all offspring of females with mixed paternity clutches, we can directly consider the relationship between the relative proportion of stored sperm from rival males in each of a female's sperm storage organs and the achieved paternity success of each male.

Chapter 4 provides information on sperm storage in wild yellow dung flies, data that are lacking even for this model system of postcopulatory sexual selection. We capture wild females at different stages of the spring season and genotype the sperm from their spermathecae to study temporal changes in sperm transfer, sperm storage and sperm competition intensity, plus prevalent levels of polyandry in a natural population.

Chapter 5 presents an oviposition site choice experiment in the field. I provide female yellow dung flies with access to three different micro-environments on a dung pat, then genotype all offspring and the sperm remaining in the spermathecae after oviposition. I test how the environment (e.g. temperature) influences egg placement, whether the number of males detected in the spermathecae and the number of sires that contribute to a clutch differ, and most importantly, I look for evidence of adaptive sperm selection (e.g. whether biases in paternity depend on the environment).

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Chapter 1

Reproductive traits: evidence for sexually selected sperm

Oliver Y. Martin and Marco Demont

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Sperm exhibit extraordinary morphological divergence yet precise evolutionary causes often remain elusive. A quantitative genetic study sheds light on the major role postcopulatory sexual selection could play in determining sperm size.

Sexual selection arises because individuals vary in reproductive success [1]. This variation often exceeds that in survivorship and sexual selection is thus a potentially powerful evolutionary force [2,3]. Classically, sexual selection is viewed as comprising of competition between males for access to females and mate choice exerted by choosy females. Disregarding direct material benefits, such mating preferences are thought to be driven and maintained by processes involving ‘good genes’ and/or ‘sexy sons’. In the former, indirect benefits arise because females prefer male traits indicative of broad genetic quality and hence produce sons (and possibly daughters) of higher viability. Under a sexy son rationale, indirect benefits stem from the production of sons with enhanced mating success. Importantly, in both good genes and sexy son processes, female mating preferences spread and are maintained through becoming genetically associated with male fitness alleles for higher viability and/or mating success. Although these processes were both incorporated in Fisher’s original verbal model [4] and genetic covariance between trait and preference features in good genes and sexy son sexual selection, the term ‘sexy son’ is usually used to describe the self-reinforcing effect resulting from genetic correlation (the Fisher ‘runaway’ process).

Sexual selection goes beyond precopulatory processes, as securing mates is not sufficient to determine reproductive success [2]. After copulation, male ejaculates compete for fertilization (sperm competition) and preferences for ejaculate traits may be expressed via the female reproductive tract (cryptic female choice). For these postcopulatory sexual selection preferences could similarly be driven by good genes (via male sperm competitive ability: good sperm [5]) or sexy sperm mechanisms (via enhanced fertilization success [6]). The ‘sexually selected sperm hypothesis’ [7,8] proposes that postcopulatory sexual selection selects for male traits that increase fertilization efficiency and female traits that promote sperm competition (multiple mating, complex female reproductive tracts). This hypothesis [7,8] includes the sexy sperm mechanism — enhanced fertilization success without enhancement of other fitness-related traits — that can lead to Fisherian runaway sexual selection. It does not exclude the possibility that overall genetically superior males have greater fertilization efficiency (the good sperm mechanism) [5,7]. A recent study [9] has investigated the impact of these mechanisms on sperm length and found support for a sexually selected sperm process.

Thorough investigation of sexual selection mechanisms requires a detailed knowledge of the specific male and female reproductive traits involved and their underlying genetics. Reproductive traits involved in mating and fertilisation are known to be subject to rapid and divergent evolution. This rapid divergence has been demonstrated in reproductive traits at various levels, from reproductive proteins and organs to behaviour [10–12]. Sperm are particularly notorious for interspecific diversity in form and size. This divergence could potentially herald strong sexual selection and has been argued to be driven primarily by male–female interactions, via postcopulatory sexual selection and/or sexually antagonistic coevolution. Rapid diversification of reproductive traits via male–female coevolution, strengthened through correlated responses in life history traits, has far-reaching implications and may impact on reproductive isolation between populations and ultimately speciation. Comparative studies on insects suggest strong associations between male and female reproductive traits, and sperm size in particular has been found to correlate with dimensions of female sperm storage organs and/or ducts [13,14].

This pattern of correlated evolution indicates that females are strongly involved in shaping sperm traits, and this possibility has also been addressed experimentally using artificial selection. Miller and Pitnick [15] created *Drosophila melanogaster* lines where males and females were selected for dimensions of key reproductive traits (sperm and seminal receptacles, respectively). Reproductive traits responded successfully to directional selection imposed as in similar artificial selection experiments [16]. Additionally, selecting for longer seminal receptacles induced a correlated increase in sperm length. Longer sperm was found to out-compete short sperm when competing in females selected for long seminal receptacles. Subsequent work [17] has provided a clear proximate mechanism for this fertilisation advantage — the heads of longer sperm are closer to the exit of the sperm storage organ and hence in better position to achieve fertilisation. Together, this research suggests that sperm length may be sexually selected. This finding of a genetic correlation and hence a possible Fisher runaway process may also help explain giant sperm in some *Drosophila* species.

Addressing similar issues in a different species, Simmons and Kotiaho [9] applied a quantitative genetic approach to the dung beetle *Onthophagus taurus*. They found significant additive genetic variation in spermatheca size and significant heritability. Importantly, consistent with sexy sperm and good sperm processes, the study shows that there is a significant negative genetic correlation between spermatheca size and sperm length: fathers that sired sons with short sperm also sired daughters with large spermathecae. Here, large sperm storage organs are genetically associated with short sperm and this is in contrast with the pattern found in *Drosophila* [15].

These results acquire further significance when placed in the context of previous findings in *Onthophagus*. Shorter sperm were found to have a fertilization advantage in competitive situations, and this advantage depended on spermatheca size [18]. Sperm length, like spermathecae size, exhibited significant additive genetic variance due to sires and, interestingly, males in better condition produced shorter sperm [19]. As a result of the genetic covariance between sperm length and male condition, females fertilizing their

eggs using shorter sperm could produce offspring of better condition (the good sperm mechanism). Taken together, these findings [9,18,19] suggest a sexually selected sperm process incorporating a (good sperm) mechanism to produce high-quality offspring. Simmons and Kotiaho argue [9] that postcopulatory sexual selection could thus shape sperm just like precopulatory female preferences affect evolutionary divergence of male secondary sexual traits [1].

This new study [9] provides compelling evidence that postcopulatory sexual selection can shape reproductive traits (particularly sperm cells), yet questions remain. Addressing these would help increase our understanding of the sexually selected sperm process in this system. Whereas in *Drosophila* the proximate mechanism for the fertilization advantage of long sperm is resolved —the sperm head is closer to the site of fertilisation) [15,17] — this is unclear for *Onthophagus*. What are the characteristics of short sperm that contribute to fertilization success? Or approaching the problem from another angle, what drives the evolution of (large) spermatheca size? Larger spermathecae could promote increased sperm competition and relate to a greater propensity for polyandry. Genetic correlations between reproductive traits (sperm and spermatheca size) and male and female mating rates could be addressed experimentally. Artificial selection incorporating monandrous (no sexual selection) and polyandrous lines (sexual selection) could be applied to verify whether fertilisation efficiency increases with intensity of postcopulatory sexual selection. This approach could also aid investigate whether inclusive fitness is higher in polyandrous than in monandrous females as predicted [8]. To specifically investigate the good sperm aspect in this system, it would be necessary to investigate offspring viability in relation to father’s fertilization success. Finally, sperm number could also play a role (for example [20]), so do males with short sperm also transfer more or less sperm (depending on how costly short sperm are to produce)? Future work in this vein could help verify key predictions of sexually selected sperm processes [7,8] and further the understanding of reproductive traits central in speciation processes.

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Chapter 2

The assessment of insemination success in yellow dung flies using competitive PCR

Luc F. Bussière*, Marco Demont*, Andrew J. Pemberton, Matthew D. Hall, and Paul I. Ward

*These authors contributed equally to the work

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Abstract

In spite of considerable interest in postcopulatory sexual selection, separating the effects of sperm competition from cryptic female choice remains difficult because mechanisms underlying postcopulatory processes are poorly understood. One methodological challenge is to quantify insemination success for individual males within the sperm stores of multiply mated females to discover how insemination translates into eventual paternity. Any proposed method must be applicable in organisms without extensive DNA sequence information (which include the majority of model species for sexual selection). Here we describe the development and application of microsatellite competitive-multiplex-PCR for quantifying relative contributions to a small number of sperm in storage. We studied how DNA template characteristics affect PCR amplification of known concentrations of mixed DNA, and generated regressions for correcting observations of allelic signal strength based on such characteristics. We used these methods to examine patterns of sperm storage in twice-mated female yellow dung flies, *Scathophaga stercoraria*. We confirm previous findings supporting sperm displacement and demonstrate that average paternity for the last mate accords with the mean proportion of sperm stored. We further find consistent skew in storage across spermathecae, with more last male sperm stored in the singlet spermatheca on one side of the body than in the doublet on the opposite side. We also show that the time between copulations may

be important for effectively sorting sperm. Finally, we demonstrate that male size may influence the opportunity for sperm choice, suggesting future work to disentangle the roles of male competition and cryptic female choice.

Introduction

Postcopulatory sexual selection remains a controversial subject partly because in contrast to precopulatory male competition and female choice, most postcopulatory selection is hidden from view within female reproductive tracts. As a result, the various influences of females and their multiple mates on postcopulatory sexual selection are difficult to disentangle, which makes separating the causes and consequences of male competition and female choice challenging (Birkhead, 1998; Birkhead, 2000; Eberhard, 2000; Pitnick & Brown, 2000; Simmons, 2001). This is especially true for the phenomenon of sperm selection, a contentious form of postcopulatory mate choice in which females use the sperm of certain males when fertilizing their eggs (Simmons & Siva-Jothy, 1998; Simmons, 2001). In many systems in which females store sperm from several males, convincing demonstrations of adaptive sperm selection could lead to evidence for sexual selection based on indirect benefits (e.g., good-genes mate choice), if for example the choice occurs in the absence of apparent direct natural selection on females (Brown *et al.*, 1997; Bussière, 2002). In addition, the accumulating evidence for sexual selection via sexual conflict makes inferring the selective basis for biases in sperm storage and use even more difficult (Arnqvist & Rowe, 2005; Bussière *et al.*, 2006), and there is widespread consensus that we need more information on the mechanisms through which biases are achieved before any conclusions can be drawn on the prevalence and importance of postcopulatory female choice in driving patterns of sexual selection (Birkhead & Moller, 1998; Ward, 2007).

Recent advances in using molecular markers to assign paternity have made studying the realised effects of postcopulatory processes relatively straightforward (Simmons, 2001). However, linking paternity to the physiological mechanisms that affect it and the ultimate evolutionary causes of these mechanisms remains an elusive goal. Previously, some researchers have employed innovative techniques to track the contributions of different males to sperm in storage, including the use of transgenic lines of animals expressing green fluorescent protein (GFP, Civetta, 1999), immunocytochemical approaches (Schärer *et al.*, 2007), the radiolabelling of ejaculates (Simmons

et al., 1999) or the use of phenotypic markers (Otronen *et al.*, 1997; Hellriegel & Bernasconi, 2000). Unfortunately, phenotypic markers such as sperm length are practically inferior to genetic markers such as microsatellites, since completely unambiguous assignment often turns out to be impossible. For example, in their study of sperm storage in yellow dung flies, Hellriegel and Bernasconi (2000) were only able to assign 79% of measured sperm to either of the two rival males based on length. In addition, it is often difficult to rule out the potentially confounding influence of most labelling techniques on sperm movement. However, the same molecular methods that currently allow the assignment of paternity can also be used to advance our knowledge of events within female sperm stores. Techniques based on allelic diversity have already been used to estimate the number of males contributing to mixed sperm stores in internally fertilising species (Bretman & Tregenza, 2005; Simmons *et al.*, 2007) and have been used to quantify the relative sperm contributions in an externally fertilising fish where sperm can be trapped on nylon membranes in the water column (Wooninck *et al.*, 2000). Quantifying the relative sperm contributions of competing males with internal fertilisation (in contrast to merely checking for their presence or absence as in allele counting) would be a further improvement in documenting the prevalence and importance of postcopulatory sexual selection.

Forensic scientists have been at the forefront of developing models for predicting the genotypes contributing to mixed DNA samples (Gill *et al.*, 1998; Cowell *et al.*, 2007). In the current paper, we describe a more straightforward exercise: to obtain information on the relative contributions of sires to female sperm stores when the genotypes of all adults are known. We used standard methods for genotyping microsatellites found in the mixed sperm of multiple sperm storage organs of female dung flies (see below). We used signal strength as an index of original DNA concentrations to study the effects of male and female phenotypes (e.g. body size) on sperm storage patterns within these organs. We corrected our estimates of signal strength for the effects of other variables thought to influence PCR amplification, namely the number and mean length of alleles in a run, the length of the focal allele, the number of alleles shorter than the focal allele, and the number of alleles represented by a single peak (i.e., whether the focal individual is homozygous at that locus). Such techniques are readily transferable to other laboratories and study organisms for the purpose of studying postcopulatory sexual selection or other applications requiring the quantification of DNA within a mixed sample.

Sexual selection in yellow dung flies

Yellow dung flies, *Scathophaga stercoraria* (L.), are a model system for studying sexual selection. Males aggregate on and around dung pats to which gravid females fly in order to lay their eggs (Parker, 1970b). Although male interactions seem to drive precopulatory sexual selection (Parker, 1970a; Parker, 1970b), females appear to retain significant control over insemination (Hosken *et al.*, 2001), thanks partly to their elaborate reproductive morphology (Hosken *et al.*, 1999; Arthur *et al.*, 2008). As in many of the Diptera, female yellow dung flies have multiple spermathecae (sperm storage organs) into which males cannot directly insert sperm (Hosken, 1999; Hosken *et al.*, 1999; Simmons *et al.*, 1999; Hosken & Ward, 2000). Instead, males deposit sperm at the entrance of the three spermathecal ducts (Hosken *et al.*, 1999), arranged into a solitary singlet spermatheca on one side of the body, and a doublet pair on the opposite side. The singlet and doublet spermathecae appear to have independent musculature in live preparations (our own unpublished observations), and thus could potentially assist in sorting sperm for subsequent sperm choice during oviposition (Hellriegel & Bernasconi, 2000).

A bias in sperm storage has been demonstrated for *Dryomyza* flies (Otronen, 1997), although demonstrating a similar pattern in *S. stercoraria* has been a challenge (Otronen *et al.*, 1997). Part of the reason for this is undoubtedly that males exert a strong influence on the outcome of insemination, and there is strong selection on both copula duration, which covaries with insemination success (Parker, 1970a; Simmons & Parker, 1992; Parker & Simmons, 1994), and on the displacement of rival sperm (Parker & Simmons, 1991; Simmons & Parker, 1992; Simmons *et al.*, 1999). Furthermore, the phenotype of sperm themselves seems to exert an influence on storage patterns in a complex way (Otronen *et al.*, 1997). Nevertheless there is substantial variation in how male traits influence sperm competition success (Simmons & Siva-Jothy, 1998). This variation could be partly due to a female influence on paternity, presumably in order to favour the ejaculates of some males over others (Ward, 1998; Ward, 2000), although this premise remains controversial (Simmons *et al.*, 1996; Simmons, 2001). Compelling evidence that postcopulatory female choice occurs comes from research showing that female experience of environmental conditions influences the siring success of mates in a way that is consistent with adaptive sperm selection (Ward, 2000). Even in this well-studied system, however, exactly how sperm sorting and selection occurs is not yet clear, and the events occurring between insemination and fertilization are most often deduced

from patterns of paternity instead of direct observations of sperm movement and storage. As a consequence, the tremendous theoretical work on postcopulatory sexual selection in this species (e.g., Parker, 1974; Parker, 1992; Hellriegel & Ward, 1998) cannot yet be thoroughly evaluated because we lack strong evidence of the mechanisms of sperm storage and use within the female reproductive tract. One of the greatest impediments to understanding cryptic female choice in this and other systems relates to the challenge in identifying paternal contributions to sperm stores, and the bias in sperm storage across spermathecae.

In yellow dung flies, average P2 (the proportion of paternity assigned to the second of two copulating males) is typically reported to be near 0.8 although there is considerable variation among individuals and across studies (Parker, 1970a; Simmons & Parker, 1992; Otronen *et al.*, 1997; Simmons & Siva-Jothy, 1998; Simmons *et al.*, 1999; Hellriegel & Bernasconi, 2000; Ward, 2007). In the focal population, average P2 is reported to be slightly lower than 0.8 (i.e., 0.74 ± 0.09 in Ward, 2000), although typically within the margin of error. In addition to developing our methods for amplifying and quantifying paternal contributions to sperm stores, we sought to determine whether this typical P2 value accords with average S2 (the proportion of stored sperm assigned to the second of two copulating males) across spermathecae, i.e., is paternity broadly consistent with a fair raffle among stored sperm? We further examined which male or female phenotypic characters helped to explain any variation in S2 that could account for large variance in observed P2 in this species. In addition, we tested for general patterns in sperm storage bias consistent with either the singlet or doublet being the preferred site of storage for males having a particular phenotype. Finally, we experimentally manipulated female storage time between successive matings, a factor that has been previously shown to influence the outcome of postcopulatory sexual selection (Hellriegel & Bernasconi, 2000), and observed its effects on biases in sperm storage across spermathecae.

Materials and Methods

Animal husbandry and laboratory matings

All the flies involved in this series of investigations were F3 or F4 descendants of adults collected in the field from dung pats in Fehraltdorf, Switzerland, and reared using a standard laboratory

protocol for dung flies (Ward, 1993, Blanckenhorn et al, submitted). We used adults that had matured for a minimum of 10 days post-eclosion in all mating experiments.

We transferred single males from their housing containers to clean vials (28.5 mm diameter x 95 mm tall) and subsequently introduced a single virgin female selected haphazardly, observing the pair to ensure that copulation occurred and noting its duration. We then introduced each mated female to a second virgin male either 1 hour or 24 hours after the completion of the initial copulation, once again noting its duration. We did not provide dung in either mating arena because we wanted to study sperm storage patterns without the complications introduced by differential sperm usage during oviposition. We allowed all females to hold sperm in storage for a full 24 hours after the second copulation before freezing them at -80°C. Although mating on dung pats in the wild is typically followed quickly by oviposition, mating also occurs away from the dung (Parker, 1971; Parker *et al.*, 1993), and sperm are retained in storage between oviposition bouts. As a consequence our sperm storage time and the absence of dung during mating reflects the natural situation in at least a subset of wild females. Mating partners were chosen at random without prior screening of microsatellite genotypes.

Dissections

We isolated stored ejaculates from previously frozen females that had been dehydrated in ethanol for a minimum of 24 hours before dissection (Tripet *et al.*, 2001). We carefully removed the posterior portion of the female reproductive tract (including the common oviduct, spermathecae, accessory glands, and copulatory bursa) from the rest of the female by grasping the genital valves in forceps and tearing them from the abdomen. We separated dehydrated, and thus solidified, sperm 'pellets' from female spermathecal tissue using *very* finely sharpened dissecting tweezers viewed under a quality binocular microscope (Leica MZ-12, Leica Microsystems GmbH, Wetzlar, Germany). We took great care to extract the entire ejaculate, and although some sperm may have been missed, this quantity relative to the extracted sperm mass is likely to be trivial. Each sperm pellet was transferred separately to a buffer solution (ATL buffer from the QIAamp® DNA Micro Kit, Qiagen; see below). The three sperm pellets from each female, which each originated from a different spermatheca, were amplified and analysed separately to study the skew in sperm storage across spermathecae. In our analyses we distinguish the singlet spermatheca (regardless of the side

of the body on which it is found) from the middle and outer doublet spermathecae (Hosken *et al.*, 1999). We also measured hind tibia length of all animals as an index of body size.

Extraction, amplification and analysis of DNA

We used DNeasy® Tissue Kits (Qiagen AG, Switzerland) to extract DNA from the heads of all flies. We used a special kit designed for use with forensic amounts of DNA sample (QIAamp® DNA Micro Kit, Qiagen AG, Switzerland) to extract the potentially very low number of DNA copies from sperm pellets. We followed the recommended protocols, including adding carrier RNA to buffer AL (1 µl dissolved carrier RNA in 200 µl buffer AL), and the minimum recommended amount of elution buffer AE (20 µl) when extracting DNA from sperm pellets in order to retain the highest possible concentration of DNA. We then used the QIAGEN® Multiplex PCR Kit to simultaneously amplify four microsatellite loci: SsCa17, SsCa24, SsCa26 (Garner *et al.*, 2000), and SsCa30 (Demont *et al.*, 2008). Total PCR reaction volume for the heads was 6 µl: 1µl DNA template, 3 µl QIAGEN Multiplex PCR Master Mix, 1.4 µl distilled water and 0.6 µl microsatellite primer mix (100 µM). Total PCR reaction volume for the sperm was 24 µl retaining the mixing ratio from the heads (e.g. DNA template and all other volumes four times higher than for the heads). Cycling conditions for the heads were as follows: 95°C for 15 min, then 27 cycles of 94°C for 30 s, 60°C for 3 min and 72°C for 45 s, and finally 60°C for 30 min. Cycling conditions for the sperm DNA were the same with one modification: 30 cycles instead of 27 to allow for the lower initial template concentration. It should be noted that using these conditions large stutter bands are usually not produced. Of these four loci, one (SsCa17) was not sufficiently polymorphic in our samples to adequately correct using the procedure described below, so for the remainder of the paper we shall focus on the other three loci. PCR products were separated on a capillary sequencer (Applied Biosystems 3730 DNA Analyzer), and the output analysed using Applied Biosystems GeneMapper® software. Each PCR amplification from template DNA was performed in triplicate to check the repeatability of our observations.

Data collection

The response variable for most of the results discussed below is the relative signal intensity of a male's alleles in an amplified subsample of the DNA extracted from the sperm pellet. Once the

adult genotypes were known, we were able to select informative alleles that could provide information on the ratio of DNA concentrations belonging to each of two putative sires. We counted as informative only alleles unique to one of the males (i.e., an informative allele could be shared by neither the rival male nor the female, even though in many cases we could find very little evidence of female DNA at other loci because our dehydration and dissection successfully separated the sperm pellet from female tissue, see results below). We also avoided using data from the SsCa30 locus in which either male had only a single allele, because previous work using this locus for paternity and population genetics analyses revealed the presence of null alleles at this locus (Hosken *et al.*, 2001; Demont *et al.*, 2008). Because this locus is highly variable, we discarded data from only seven pairs of males at this locus; in all other cases both males possessed two visible alleles and their contributions to mixed sperm could not have been underestimated as a result of null alleles. Signal strength was assessed as the area of an individual peak rather than peak height because for very intense peaks we often observed a greater peak width (see Figure 1).

Correcting estimates of signal strength

Many factors besides the initial concentrations of alleles (e.g., allele length) may contribute to the observed signal strength of a particular allele after PCR (see e.g., Suzuki & Giovanni, 1996; Haberl & Tautz, 1999; Lion, 2003). We corrected measures of relative peak intensity for each of our microsatellite loci using linear mixed models of signal strength on several allele characteristics that we reasoned might influence signal strength. These models were computed using controlled mixtures of DNA from genotyped adults. We used 61 adult samples to study the effects of allele characteristics on genotyping signal strengths. These samples were chosen to cover the range of allele sizes and combinations found in the main study. For each sample, we estimated DNA concentration in the extraction twice for each of two independently drawn samples using an Eppendorf BioPhotometer (Eppendorf AG). Repeated measurements of the same subsample were highly consistent, as evidenced by a very strong correlation in estimated concentrations ($r = 0.998$, $n = 122$), and the correlation across independently drawn samples from the same individual was only slightly lower ($r = 0.991$, $n = 61$). Of the 61 samples measured, 9 had concentrations lower than 30 μg/ml, and were discarded. We diluted the remaining 52 samples to a standard concentration of 30μg/ml dsDNA. Subsequently, we haphazardly selected 96 pairings of two of these individuals that would provide information on a minimum of two loci. We mixed together the

DNA in seven ratios: 0.0625, 0.125, 0.25, 0.5, 0.75, 0.875, and 0.9375. (Our protocol for diluting the concentration for this series of mixtures produced two independent mixtures at a ratio of 0.5 for each block of eight mixtures.) This range was chosen to reflect the possible variation in sperm ratios within female spermathecae. We then observed how signal strength for different alleles varied according to initial concentration and several specific properties of the particular PCR and sequencing run. Based on discussions with colleagues and our observations of the behaviour of signal strength in heterozygotes, we included the following allele characters in our linear models: initial relative allelic concentration (from the controlled mixtures), number of alleles being amplified in the reaction, number of shorter alleles (relative to the focal allele) amplified in the reaction, mean length (in bases) of alleles in the reaction, relative length of the focal allele (focal allele length – mean allele length), and whether or not the focal allele was homozygous in the focal individual. The resulting equations could then be solved for initial allelic concentration, and the rearranged equations used to correct observed values of signal strength for experimental runs in which the starting DNA concentration was unknown.

Statistical analyses

We estimated the effect of allele characteristics on peak signal strength with mixed models (using the lme function in the nlme package for R, Pinheiro *et al.*, 2008) of arcsine square root transformed proportions of informative allele signal observed. The fixed effects included first and higher order terms for the initial DNA concentrations (based on the mixture in question) and the allele characteristics described above, while the random effects were the PCR run nested within the particular pairing of individual samples. We compared higher order models (including quadratic and cubic terms) that would allow for nonlinear changes in the observed signal strengths as the ratios of alleles contributing to the PCR run changed with first order models (allowing only linear trends) by computing the Bayesian Information Criterion (BIC). BIC is a penalized log-likelihood measure that quantifies goodness-of-fit for a model but trades-off model fit with the number of parameters included, and tends to favour simpler models than rival methods such as the Akaike Information Criterion (Burnham & Anderson, 2004). This was ideal for our purposes since we sought a simple model that predicted the effects of allele properties on signal strength, but in which the explanatory variable “initial DNA concentration” could be easily isolated from other terms in

order to convert observations of relative signal strength in experimental PCR runs to an estimate of the relative starting concentration of alleles.

After correcting signal strengths as described above, we adjusted estimates of DNA ratios if one of the males had more informative copies of DNA that the other (e.g., if the first male had two informative alleles but the rival had only one, we halved the corrected signal strength of the first male's allele before calculating the ratio in order to get a fair estimation of each male's contribution to the ejaculate stores). These data represented our indices of S2 (the fraction of sperm stored within a spermatheca that belongs to the second of two males mated to a female, which is analogous to P2 values in paternity studies). In a few cases, the corrections produced estimates of S2 that were slightly higher than 1; these estimates were adjusted to a value of 1 (i.e., complete second male priority) before proceeding. We then transformed all DNA ratio data using arcsine square-root transformations. None of these transformed S2 distributions (of data or residuals) showed any evidence of significant deviations from normality after transformation (all Kolmogorov-Smirnov tests: $p > 0.10$). Because we had up to three estimates of relative concentration from each spermatheca (from the three independently evaluated microsatellite loci), we were able to assess the repeatability of our estimates before and after correction using ANOVA (Becker, 1992). The transformed S2 ratio from all informative loci for each spermatheca was used in our subsequent assessment of factors affecting skew in sperm storage.

We used one-sample t-tests on our experiment-wide transformed S2 values against the expected values of 0.8 and 0.5 (following arcsine square root transformation), from previous findings of paternity in this species and models of sperm storage featuring no displacement, respectively. We then built linear mixed effects models as above to study the within female skew in storage across spermathecae and the between-female effects of treatment (time interval between matings) on overall S2. The fixed effects included the time interval between mating (1 hour or 24 hours), the spermathecal identity, the hind tibia lengths of the female and both males and the duration of both copulations, while the random effects were the locus for which the data were collected nested within spermatheca nested within female. To further study what influenced biases in sperm storage across the singlet and doublet (which arguably represents the opportunity for sperm selection), for each female we then calculated the difference between the singlet S2 value and the mean of the doublet S2 values. We then modelled the effects of behavioural and morphological attributes of the

females and their mates on this index of bias. In both cases, the significance of individual terms was not sensitive to the structure of the model, and consequently we present the full model including non-significant terms in our results. Statistical analyses were conducted using SPSS (Anonymous, 2005) and R (R Development Core Team, 2008).

Results

Correcting PCR runs

We conducted 768 PCRs of mixed DNA (96 pairs X 8 concentration mixes), but because not all pairings had informative alleles for all loci, the number of informative runs and peaks for each set of regressions differs. The observed ratios of alleles consistently overestimated the fraction of DNA represented by alleles present in low concentrations and conversely underestimated alleles present at high concentrations, although in general the uncorrected estimates were reasonably close to predicted values (see supplementary Table S1). We therefore used a series of linear mixed models to improve the correspondence between predicted and observed ratios of allele signal strengths. We summarize the comparisons between higher order and first order models in supplementary Table S2. In all three loci, the linear first order model had the highest BIC weight.

We summarize the parameter estimates for these linear first order models in Table 1. For all three loci, the number of alleles shorter than the focal allele and the heterozygosity of the focal allele were associated with relatively high coefficients, indicating an influence on the predicted signal strength. Heterozygous alleles had higher observed signal strength than half the predicted value of a single homozygous allele, while a greater number of shorter alleles dampened the observed signal strength. The total number of competing alleles typically had a small coefficient, but for SsCa24 large numbers of alleles seemed to slightly increase predicted signal strength. Relative allele length was associated with only small parameter estimates in all three loci.

Repeatability of competitive PCR

In the main study we attempted to mate 60 females to two males each (30 for each sperm storage time treatment). Of these 60, two females failed to mate with one of their assigned mates and an

additional four females had four spermathecae (as is found in a small fraction of wild-type flies, see Ward, 2000). All were removed from the analysis to simplify its interpretation. A further ten females were removed from the analysis because one of the three specimens in a mating triad were lost before DNA extraction (n=2) or because of failures in removing all three spermathecae without damaging them (n=8). The remaining 44 females were evenly split amongst the two sperm storage time treatments.

Even among the subset of preparations for which our dissection notes did not indicate any obvious contamination by female tissue (n = 38), there was sometimes evidence of female DNA in the fragment analysis runs (in 15/38 cases, female contributions to peak areas exceeded 10% of the total signal area for a genotyping run). Almost all of these samples were conducted early in the sequence of dissections, and our ability to remove contaminating female tissue improved over time (in the last samples we find nearly no contaminating female tissue at all). In any case, the magnitude of female contamination was usually rather low (mean 12.0 ± 2.4% of signal in a PCR run).

The relative signal strengths of alleles from the same locus across replicate PCR and genotyping runs were highly repeatable (repeatability = 0.91). This confirms that for a given mixed sample of DNA, our PCR conditions were sufficiently consistent to provide a reliable estimate of the original DNA concentration. However, the uncorrected estimates obtained across different loci within the same spermatheca were less consistent (repeatability = 0.71), indicating that the particular characteristics of a competitive PCR run play a substantial role in determining the correspondence of signal strength to starting DNA concentrations. By correcting our estimates of signal strength using equations derived from the parameter estimates described in Table 1, we were able to increase the repeatability across loci to 0.77. Results from tests based on a single locus were qualitatively the same as those for multiple loci. The loss of power was attributable to the fewer degrees of freedom afforded analyses of individual loci compared with the model in which locus was nested within spermatheca. All of the results reported below are therefore those for the corrected dataset using all three loci.

Sperm storage patterns in doubly mated yellow dung flies

One-sample t-tests revealed that our experiment-wide findings of S2 averaged across spermathecae were significantly different from 0.5 as would be expected in a situation without sperm displacement ($t = 5.883$, 43 df, $p < 0.001$; see Figure 2 for mean S2 values across treatments). By contrast, these same mean values were not significantly different from 0.8 ($t = -0.837$, 43 df, $p = 0.407$), supporting previous reports that sperm displacement occurs during copulation, and that on average for this species, paternity is assigned in proportion to the relative number of sperm in storage. However there was considerable variation in both the overall level of sperm storage priority accorded for second males, and also substantial variation in the patterns of storage across the spermathecae.

To explore this variation, we modelled changes in S2 across spermathecae as summarized in Table 2. Several sources of variance significantly explained variation in S2 across spermathecae and across females. The significant effect of spermatheca indicates a consistently lower S2 estimate for the doublet spermathecae compared with the singlet (see Figure 2). The duration of the second male's copula (in seconds) has a significant positive effect on S2 across spermathecae, ($\beta = 0.0001052 \pm$ SE 0.0000363, $p = 0.0063$). As in a previous analysis of paternity (Parker & Simmons, 1991), the copula duration of the first male did not contribute significantly, nor did the size of the female or of either male. We detected a significant interaction between spermathecal identity and the time interval between matings. This interaction indicates a significant effect of sperm storage time on the skew in S2 across spermathecae: when females are given 24 hours between matings, the difference in S2 value between the singlet and middle doublet is more pronounced than it is when the second male mates only one hour following the first (see Figure 2).

This mixed effects model examined systematic skew in sperm storage patterns across all three spermathecae. However, we were also interested in exploring sperm storage skew across the singlet and doublet specifically, as this might represent the opportunity for choice given independent female control over the singlet versus the doublet spermathecae. We computed the difference between the singlet and the mean S2 for the doublet and used a univariate analysis to explore covariance between this difference and the time between matings as well as behavioural or morphological covariates (see Table 3). The only significant contributor to changes in the skew

across the singlet and doublet spermathecae was the time interval between matings, with greater skew occurring when copulations were separated by 24 hours than when they were only one hour apart (1 hour skew: 0.043 ± 0.033, 24 skew: 0.132 ± SE 0.024). The hind tibia length of the second male exerted a marginally non-significant effect, in which larger second males tended to promote greater skew in sperm storage ($\beta = 0.104 \pm$ SE 0.050).

Table 1 Summaries for first order linear regressions of observed signal strength on various allele properties for three microsatellite loci (Garner *et al.*, 2000; Demont *et al.*, 2008) in yellow dung flies. The three original equations for correcting observed proportion signal strength were based on the original parameters, but the rearranged equations were used to calculate the initial DNA concentration (thus the coefficients in the equation are the inverse of the parameter estimates listed below). For example, the rearranged equation for SsCa24 is as follows: transformed initial [DNA] = -0.074 + 1.122*observed proportion signal + 0.084*no. shorter alleles – 0.006*relative allele length – 0.016 * heterozygosity – 0.019*no. alleles in run.

Locus	No. of informative mixtures (/96)	No. of informative PCR runs	Regression parameter estimates for original equations					
			Intercept	Transformed initial DNA concentration	No. of shorter alleles in run	Relative allele length	Focal allele heterozygosity	Total no. of alleles visible in run
SsCa24	79	594	0.066	0.891	-0.075	0.005	0.015	0.017
SsCa26	65	487	0.050	0.909	-0.033	-0.001	0.040	-0.008
SsCa30	86	642	0.019	0.939	-0.032	-0.002	0.033	0.000

Table 2 Summary of the linear mixed model for transformed proportion of second male sperm in storage as a function of the time interval between matings, the spermatheca, and behavioural and morphological covariates. The model included the locus that provided a given estimate nested within the spermatheca nested within the female as random effects.

Source	Numerator df	Denominator df	F value	*p* value
Time interval between matings	1	37	0.0005	0.9829
Spermatheca	2	84	9.7821	0.0002
Time interval X Spermatheca	2	84	3.7976	0.0264
Female hind tibia length	1	37	2.6761	0.1103
1st male hind tibia length	1	37	1.2675	0.2675
2nd male hind tibia length	1	37	0.0690	0.7943
1st male copula duration	1	37	0.5250	0.4733
2nd male copula duration	1	37	8.4056	0.0063

Table 3 Analysis of variance of the effect on time between mating and morphology on the difference in S2 values between a female's singlet spermatheca versus her doublet spermatheca.

Source	df	MS	F value	*p* value
Time interval between matings	1	0.0863	4.708	0.037
Female hind tibia length	1	0.0012	0.066	0.798
1st male hind tibia length	1	0.0002	0.008	0.928
2nd male hind tibia length	1	0.0687	3.746	0.061
1st male copula duration	1	0.0072	0.393	0.534
2nd male copula duration	1	0.0098	0.532	0.470
Error	37	0.0183		

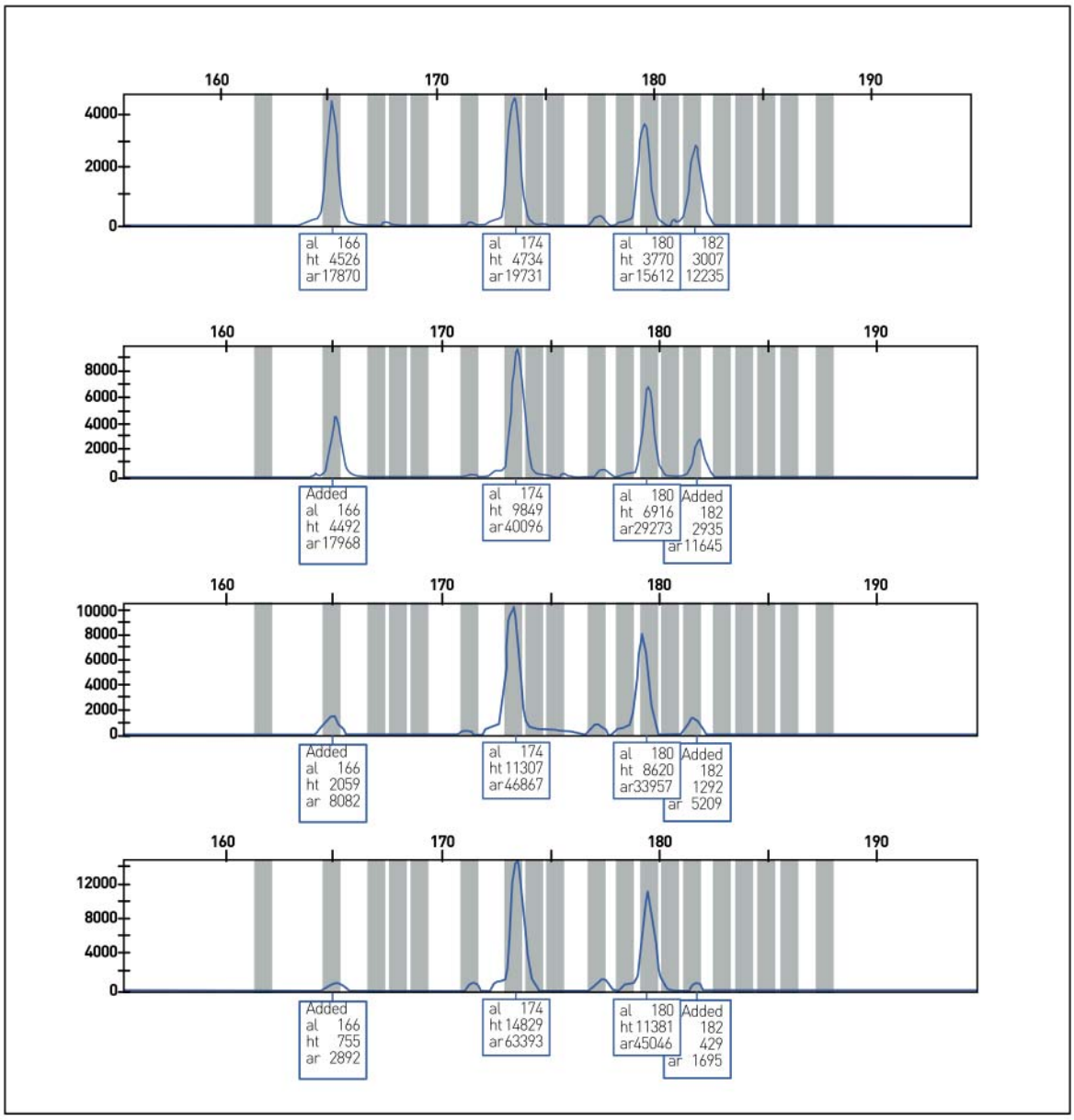

Fig. 1 Electropherogram from ABI GeneMapper software illustrating how we assessed peak intensity in four different PCR reactions. DNA from two heterozygous genotypes (one having alleles 174 and 180 for locus SsCa30, and the other having alleles 166 and 182), were mixed together in four different ratios, shown from top to bottom of the figure as follows: 1:1 (mimicking an S2 for the pair above of 0.5), 3:1 (S2 = 0.25), 7:1 (S2 = 0.125), and 15:1 (S2 = 0.0625). The peaks under the line illustrate signal strength for each of the alleles (possible alleles for this locus are shaded in light grey), and the signal strength can be quantified using peak height or area (denoted by ht and ar, respectively, in the box below each peak).

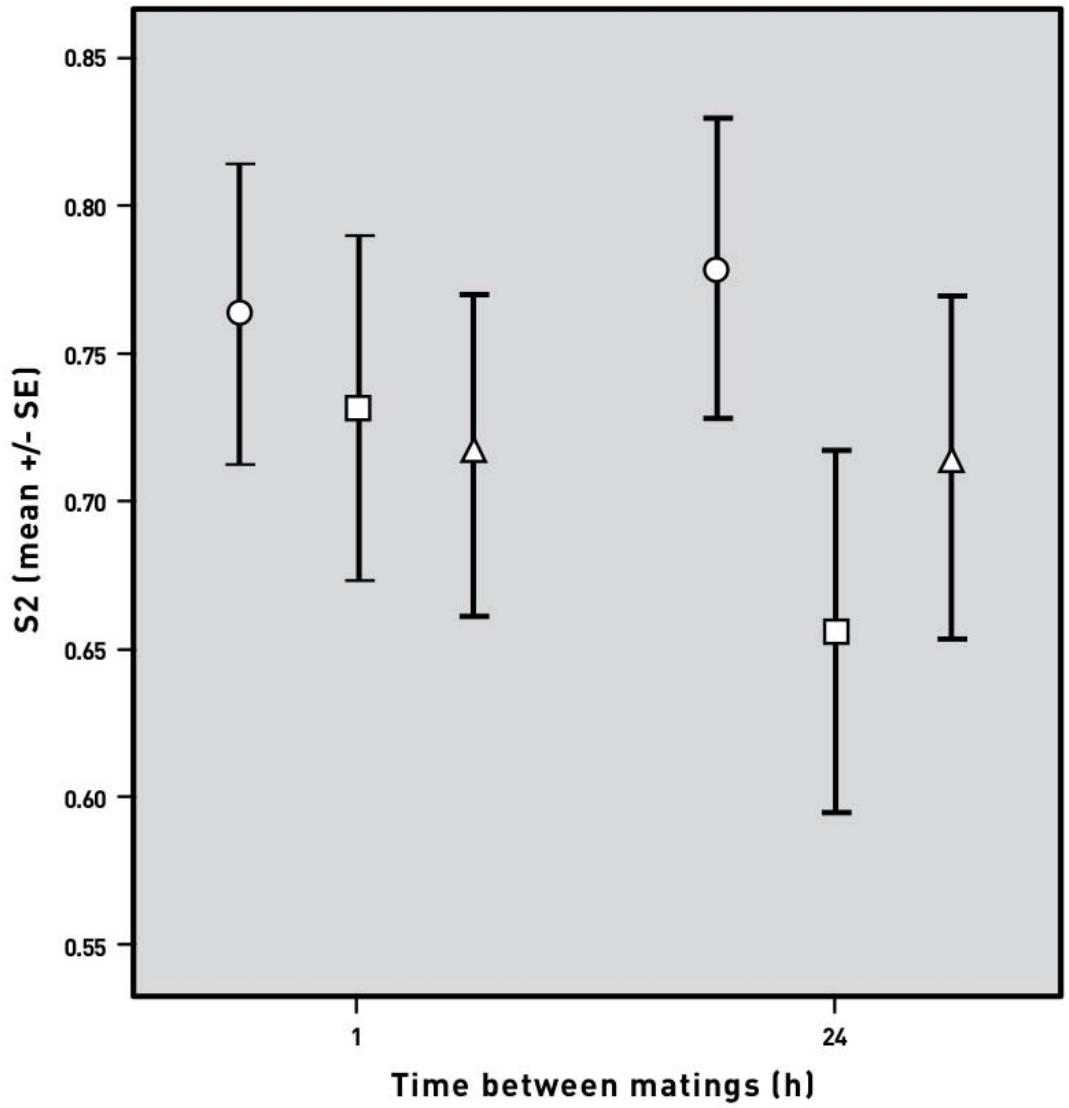

Fig. 2 The mean (±SE) proportion of second male sperm in storage in each of the three spermathecae (circles: singlet spermatheca; squares: middle doublet spermatheca; triangles: outer doublet spermatheca) as a function of the number of hours (experimentally designated as either 1 or 24) elapsed between matings.

Discussion

Sperm storage in yellow dung flies

In the present study we successfully employed competitive PCR to study biases in sperm storage across the sperm storage organs of female yellow dung flies. As a broad measure of second male success, we found that overall S2 across spermathecae was consistent with the general pattern of last-male paternity (P2) in this species, which confirms previous findings suggesting sperm displacement by the last male (Parker, 1970a; Parker & Simmons, 1991; Simmons *et al.*, 1999) over alternatives such as stratification within the sperm stores of females. We note that P2 can be highly variable within this species, ranging from complete first male precedence to complete last male precedence (Otronen *et al.*, 1997; Simmons *et al.*, 1999; Hellriegel & Bernasconi, 2000), and that we also found substantial variation in S2 that is as yet unexplained. We encourage future work that directly links the empirical observations of contributions to stored sperm within the spermathecae and paternity.

The significant spermathecal identity effect (Table 2) supports consistency across females in the pattern of sperm storage for the second male, which accords with previous work on a dryomyzid fly (Otronen, 1997). Our observations of skew across spermathecae in S2 values, with the singlet typically having higher S2 values than in either doublet spermatheca (Figure 2) imply one of two scenarios, which are not mutually exclusive: 1) there is a level of consistency in female influence on the patterns of sperm storage; or 2) the second males consistently fill spermathecae in the same order, with the singlet being filled first. However, this second scenario would predict an interaction between the second male's copula duration and spermathecal identity, which our data do not support. While this failure to detect an effect could conceivable be a result of low power (we cannot rule out that such an interaction would require more sensitive techniques than the one employed here), there is no trend in the data suggesting that an increase in sampling would produce a significant interaction.

In *Dryomyza anilis*, males use their abdominal claspers to tap the females' abdomens during copulation, and more sperm move into the singlet spermatheca when this behaviour occurs. Furthermore, females preferentially use sperm from the singlet during oviposition, so males who tap

more gain an advantage over rivals (Otronen, 1997). In *S. stercoraria*, previous work revealed a more complex relationship between male size, sperm morphology, mating order, and the bias in sperm storage which did not suggest a single consistent site of storage for preferred sperm (Otronen et al, 1997). Our results suggest that a consistent bias may be observed in *S. stercoraria* under some conditions. If this is true, one might predict that the contents of the singlet and doublet spermathecae are not equally likely to be used during fertilization. Alternatively, the presence of multiple sperm storage organs containing different mixtures of competing ejaculates may allow females more precise control over paternity even if all sperm stores are used equally. In this case, the sorting that occurred during copulation would be critical in defining the relative success of different males. A final intriguing possibility is that sperm are segregated in order to sort sperm by age, although there is no evidence that sperm function declines with age (Bernasconi et al., 2002). More work on the relative contributions of each sperm store to fertilization will be needed to address these questions.

Unlike the study by Otronen et al (1997), our analysis does not suggest any other phenotypic male character that relates to this skew, but we acknowledge that we measured only a single aspect of male morphology, and that our study was primarily designed to observe the effect of storage time between matings rather than male phenotypic characters. The significant interaction between the time interval between matings and spermathecal identity supports a role for females in sperm sorting, although the extent to which this pattern is the result of adaptations specifically evolved in the context of mate choice remains an open question. In nature, sperm are stored between oviposition bouts that can be separated by weeks, but male contests on the oviposition resource can often result in successive copulations separated by mere minutes, typically followed immediately by a bout of oviposition (Parker, 1971). Although our experimental flies were prevented from ovipositing, our results nevertheless suggest that in the latter instance, sperm storage may be less skewed, and from the perspective of the last mate, therefore less at risk of any female sperm choice that counteracts the typical last male advantage in this species.

We note that the average difference in S2 across spermathecae represents one potential aspect of sexual selection, but does not capture the opportunity for individual females to exercise choice if the doublet spermathecae operate as an integrated unit. Our second analysis therefore reduced the S2 values in the three spermathecae to the difference in S2 within the singlet and the doublet, with

large positive values indicating relative high S2 in the singlet and vice-versa. Once again, the time interval between matings influenced the skew across the singlet and doublet spermathecae, but in addition the model included a marginally non-significant effect of the second male's hind tibia length, suggesting that the opportunity for exercising sperm choice is greatest when there is more time between matings and when the second male is large. While clearly tentative given its nonsignificance, the effect of male size in this instance deserves more study, as it is consistent with both female preferences for large males or alternatively size-dependent differences in success in sperm transfer that occur in the absence of active female mate choice.

Our results, which do not suffer from many of the technical shortcomings associated with previous methods for estimating relative contributions to sperm stores (see Introduction), contribute to increasing evidence that females may have a role in biasing sperm use in yellow dung flies. An obvious next step in understanding the mechanisms affecting sperm sorting and fertilization is to relate patterns within sperm stores to eventual paternity. For example, separately amplifying portions of the sperm pellet (i.e. proximate to or distal from the spermathecal duct) could provide a thorough test for sorting within individual sperm stores. Because our methods are invasive (involving sacrificing the females in question) the necessary experiments will require careful consideration of events occurring between sperm storage and fertilization. Such work will doubtless clarify the intricate coevolutionary relationships underlying the remarkable animal diversity in reproductive morphology and biochemistry.

Methodological findings

Our approach to quantifying mixed DNA does not depend on an assumption that all alleles amplify equally well, but rather that allele characteristics affect amplification in a predictable way. Even before correcting the ratio of signal strength for allele properties, the signals provide reasonably accurate estimates of the starting concentrations of DNA (see Table S1). Correcting raw scores of signal strength using observations of allele amplification in controlled mixtures substantially improved our ability to replicate estimates of S2 across different loci (repeatability across loci increased from 0.71 to 0.77 after correcting for allele properties). Our methods are sufficiently well resolved to study variation in sperm storage within a single female in spite of the fact that we have restricted our correction to a linear model, the unexplained variation in signal amplitude across loci,

and the relatively low number of sperm copies obtainable from individual sperm storage organs of female dung flies. This repeatability may be insufficient for some applications, but we note that when specimens are not as heavily affected by sampling error, the repeatability may increase. Ongoing work that has adapted the protocol for crickets has been very successful and demonstrated higher repeatabilities of up to 0.82 across loci and 0.96 across replicate PCR runs (Hall et al., unpublished). Many model systems have a large number of microsatellite markers available, and using a larger number of loci should also increase the accuracy of the estimates, although we were unable to test this given the small number of loci we studied. Our method does not required the extensive genomic knowledge that would be needed to develop a suitable array of SNP markers for real-time PCR (e.g., Wilkening *et al.*, 2005), nor is it limited to documenting gene presence / absence conditions as for example used in XY-FISH protocols for quantifying chimerism after cell transplantation (Buño *et al.*, 2005). As portfolios of microsatellite markers are now developed for a large number of species, these techniques have the potential to be widely applied.

We note that correcting for allelic characteristics using controlled mixtures is time-consuming and associated with moderate costs. Furthermore, as is evident in differences across loci within this system, these corrections will need to be done independently every time a new series of markers are to be used for quantifying the relative DNA contribution to a mixed sample. It may also be the case that the relationship between specific characteristics of an allele and the signal strength observed changes across different instruments in different laboratories, and so we advise basic good laboratory practice, where all measures of controlled mixtures used for correcting estimates of allele areas be conducted on the same instruments with the same reagents as those used for amplifying and assessing the mixtures of interest themselves.

We restricted our analyses to instances in which the contribution of a male could be unambiguously inferred because the male possessed a unique allele (not shared by the other male or by the female) at that locus. In principle, it would be possible to estimate male contributions by subtracting the estimated female contamination (on the basis of female alleles that are unique at the same or a different locus). It may also be possible to combine information from multiple loci to infer male contributions to peaks shared by the males themselves. In this first application of our newly developed methods, we wanted to keep the analysis as simple as possible given the number of alleles to which we had access. The necessity to exploit all the information available from

amplifications of mixed DNA will depend on the number of loci available, their level of polymorphism, and the confidence with which researchers can rule out female contamination of samples. In our study, the fact that in early dissections we were inconsistently able to completely isolate sperm from female tissue (although we did improve in this skill over time) and the availability of alternate loci at which contributions could be unambiguously assessed suggested a conservative approach.

We hope that these statistical methods for assessing contributions to mixed sperm stores will be useful in other contexts in addition to studies of postcopulatory sexual selection. The wide availability of microsatellites for many study systems makes this approach feasible across a wide range of organisms, and the limited technical requirements and relatively low-cost of fragment analysis would allow its implementation for a number of applications, including for example the assessment of the fraction of self-fertilizing pollen on the stigmata of plants, determining the ploidy level of individuals, the diet composition of planktivores and bacteriotrophs, and measuring the success of different parasitic strains within individuals.

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Chapter 3

How biases in sperm storage relate to sperm use during oviposition in female yellow dung flies

Marco Demont, Paul I. Ward, Wolf U. Blanckenhorn, and Luc F. Bussière

Abstract

Mechanisms underlying sperm storage and utilization are largely unknown, and data that directly link the number of stored sperm to paternity are extremely scarce. This lack of data has seriously impaired a critically assessment of male and female influences on differential fertilization success. We used competitive microsatellite PCR to study the effects of oviposition, dimensions of the female reproductive tract, body size and copula duration on the proportion of stored sperm that were provided by the second of two copulating males (S2) in yellow dung flies, *Scathophaga stercoraria*. We genotyped all offspring from potentially mixed-paternity clutches to establish the relationship between stored sperm (S2) and paternity success (P2) of the second male. We found consistent skew in sperm storage across the three spermathecae, with more second male sperm stored in the singlet spermatheca than in the doublet. S2 values generally decreased with increasing spermatheca size, indicating less efficient sperm displacement in large spermathecae. Additionally, copula duration and several two-way interactions that included spermatheca identity, female size, and size of the second male significantly influenced S2, highlighting the complexity of postcopulatory processes and sperm storage. Importantly, S2 and P2 values were overall strongly correlated: 0.902, singlet spermatheca; 0.863, middle doublet spermatheca; 0.836, outer doublet spermatheca. Oviposition-treatment strongly influenced S2, with S2 being smallest when females laid their eggs after the second copula. Interestingly, and contrary to prediction, S2 values were higher when females did not lay eggs than when they oviposited between copulations. Our study therefore revealed that many factors influence sperm storage, but that the proportion of stored sperm is strongly linked to paternity, an observation that is most parsimoniously explained by largely random sperm utilization. Nevertheless, substantial unexplained variance could reflect a certain degree of sperm choice by females. We argue that many more data such as these will

be needed to reveal the mechanisms mediating nonrandom paternity, and to assess the importance of sperm competition and cryptic female choice for differential fertilization success.

Introduction

An extensive body of literature documents the patterns of sperm utilization following double matings, and both first male and last male priority in gaining fertilizations have been observed (Simmons 2001). In addition to mating order, several factors including copula duration (Parker & Simmons 1994), rate of sperm transfer (Parker & Simmons 2000), sperm morphology (Garcia-Gonzalez & Simmons 2007), female reproductive tract morphology (Fedina & Lewis 2004), mating interval (Cochran 1979) and the time between mating and oviposition (Ueno & Ito 1992) can all strongly affect paternity outcomes. Unfortunately, assessing the relative importance and evolutionary implications of these factors has been impaired because the processes controlling fertilization success are usually inferred from patterns of paternity without any information about how sperm are transferred, stored and used. Such information is essential for interpreting the causes and consequences of broad patterns in paternity after multiple mating (Lessells & Birkhead 1990; Parker et al. 1990; Simmons 2001). Without more information about the mechanisms mediating fertilzation success, estimating postcopulatory selection is difficult, and assessing the relative importance of male (sperm competiton) and female (cryptic female choice) processes, as well as the interactions between them, for differential fertilization success is impossible (Birkhead & Pizzari 2002; Snook 2005).

Sperm competition is indisputably an important evolutionary force (Parker 1970c). Sexual selection via sperm competition has driven the evolution of many male behavioural, physiological, and morphological traits involved in the avoidance or engagement in competition for the fertilization of a set of ova (Birkhead & Moller 1998; Simmons 2001). Additionally, sperm competition has important implications for life history evolution and speciation. In contrast, the role of females in determining fertilization outcomes and driving evolution has received relatively little attention. This imbalance is glaring, because females provide the selective environment in which postcopulatory sexual selection occurs, and they are certainly not passive agents in the process (Lloyd 1979; Parker 1970c). Thornhill (Thornhill 1983; Thornhill 1984) introduced the term cryptic female choice to describe female

processes occurring during and/or after copulation that bias paternity or offspring production toward a certain male. In subsequent decades, more than 20 potential mechanisms enabling females to exert cryptic choice have been described (Eberhard 1996). Fifteen kinds of these mechanisms seemed applicable to insects, categorized into the following five subgroups: female influence on remating, sperm transfer, sperm storage, sperm utilization at the time of fertilization (i.e. sperm selection), and investment in offspring (Eberhard 1996; Eberhard 1997; Simmons 2001). Although a female influence during any one of the postcopulatory stages could easily have a large impact on the fertilization success of a particular male, such influences are poorly documented and some proposed mechanisms of cryptic female choice (e.g. sperm selection) have so little empirical support that their importance remains doubtful (Birkhead 1998; Simmons 2001). This paucity of evidence is potentially explained to a certain degree by the fact that empirically examining these processes within females is very challenging. Additionally, some of the techniques that have been used to study these phenomena suffer from practical limitations (Birkhead 2000; Bussiere et al. 2009). For example, quantifying sperm in storage with phenotypic markers such as sperm length is often difficult, because complete univocal assignment is impossible (Hellriegel & Bernasconi 2000). Furthermore, sperm size may not be selectively neutral with respect to fertilization success, which could easily obscure the relationship between insemination success, sperm storage and paternity. Newly developed molecular methods such as competitive microsatellite PCR (Bussiere et al. 2009; Hall et al. 2010) avoid some of the limitations of earlier approaches, and therefore promise to promote our understanding of the mechanisms mediating sperm competition and cryptic female choice.

One of the model systems for sexual selection is the yellow dung fly, *Scathophaga stercoraria*, which has been the focus of many studies using a rich array of approaches (e.g. field studies, laboratory mating trials, and experimental evolution). Male interactions seem to drive precopulatory sexual selection (Jann et al. 2000; Parker 1970a; Parker 1970b), but females appear to retain some control over postcopulatory processes (Ward 2007). As in many of the Diptera, female yellow dung flies have multiple sperm storage organs (spermathecae) into which males cannot directly insert sperm (Hosken 1999; Hosken et al. 1999; Hosken & Ward 2000; Simmons et al. 1999). Instead, males ejaculate into the bursa copulatrix (Hosken 1999; Simmons et al. 1999), with the phallosome (endophallus) almost directly abutting the spermathecal duct openings (Hosken et al. 1999). Female yellow dung flies have three spermathecae (one called the singlet on one side of the body, and a pair

collectively called the doublet on the opposite side), each with its own narrow duct (Hosken et al. 1999). Several lines of evidence suggest a possible role for these organs in sperm choice. First, theoretical work has shown that separate sperm stores could allow differential storage rates (e.g. transport to the spermathecae) and differential use for different males (Hellriegel & Ward 1998). This theoretical work is complemented by observations that the singlet and doublet spermathecae have independent musculature in live preparations (L. F. Bussière, unpublished observations), and thus could potentially assist in sorting sperm for subsequent sperm selection during oviposition (Hellriegel & Bernasconi 2000). Recent findings clearly demonstrated that sperm contents differ amongst spermathecae following double matings in the laboratory (Bussiere et al. 2009), and across spermathecae of wild-caught females (Demont *et al.*, Chapter IV and V), but whether these biases may enable adaptive sperm choice remains unclear.

Another line of evidence, the finding that females stored more sperm from larger mates, was initially interpreted as support for female preference for sperm from large males (Ward 1993). However this pattern is also consistent with large males having a higher rate of sperm transfer and displacement than small males in the absence of active mate choice (Parker & Simmons 1994; Parker & Simmons 2000; Simmons & Parker 1992). In the context of exploring mechanisms of sperm transfer and storage, Simmons *et al.* (1999) demonstrated that the pattern of sperm storage within spermathecae is best explained by indirect volumetric displacement of previously stored sperm (i.e. females facilitating the exchange of sperm from the bursa copulatrix to the spermathecae) coupled with immediately random sperm mixing. Following sperm displacement and mixing, Simmons (2001) assumed that sperm are used at random from the spermathecae without sperm selection. In this species, average S2 (the proportion of stored sperm assigned to second of two copulating males) accords with average P2 (the proportion of paternity assigned to the second of two copulating males), supporting the idea that paternity is a function of the relative number of sperm in storage (Bussiere et al. 2009).

These observations notwithstanding, data that directly link the proportion of stored sperm within the spermathecae to paternity are still missing. Furthermore, there are several reasons that the notion of a predominantly male-driven pattern of sperm use, without any active influence by females, is likely to provide an inadequate account of fertilization success in this species. First, the development and maintenance of the complex female reproductive tract

with three spermathecae requires energy (e.g. implies costs), and therefore suggests an adaptive advantage. Given that female dung flies are probably never sperm-limited, the most obvious explanations rely on some form of cryptic female choice. Second, recent work clearly demonstrates consistent biases in sperm storage across the spermathecae that are required for sperm selection but difficult to explain by resorting only to intrasexual selection (Demont *et al.*, Chapter IV and V) (Bussiere et al. 2009). Third, mean P2 in the laboratory and P_{last} (the proportion of paternity assigned to the last of several copulating males) for field data is usually approximately 0.80, but the variance around this mean is enormous (P2 ranges from 0.02 to 1), and often unexplained (Simmons & Siva-Jothy 1998). Finally, there is evidence that female yellow dung flies are able to bias paternity toward certain males depending on environmental conditions (Ward 2000), which clearly suggests that females can have some influence over paternity despite sperm displacement. These arguments emphasize the uncertain role of females in affecting fertilization even in this well-studied system.

One glaring gap in the study of sperm usage in yellow dung flies is that we have no direct correlations between biases in sperm storage and skew in paternity. The present study addresses this issue directly. We specifically relate biases in paternity to patterns of sperm storage by manipulating the occurrence and timing of oviposition relative to double matings in a controlled laboratory experiment.

We had the following expectations: (1) Oviposition between the two matings (i.e. use of sperm from the first male) will result in an increased proportion of stored sperm assigned to the second male. (2) Further, if sperm are largely used at random during fertilization (i.e. in the proportion they are present within sperm stores), the proportion of stored sperm assigned to the second male should be similar for flies without oviposition and flies that immediately oviposited after the second copula. (3) Additionally, largely random sperm utilization (i.e. no sperm selection by females) would also be reflected in a strong correlation between the amount of stored sperm and achieved paternity success of the males.

Materials and Methods

Animal husbandry and laboratory matings

All the flies involved in this experiment were F1 descendants of adults collected in the field from dung pats in Fehraltorf, Switzerland and kept in the laboratory under following

conditions: 22°C, 60 % relative humidity and 12 h photoperiod with water, sugar, and *Drosophila melanogaster* as prey supplied ad libitum.

All 105 females in the experiment were randomly assigned to one of three mating-oviposition treatments (35 females per treatment): two copulations without oviposition; oviposition between mating one and mating two, and oviposition after the second copulation. All females were mated with two different males and copulations in all treatments took place on consecutive days, so that mating interval (which is known to affect sperm storage patterns in yellow dung flies; Bussière et al. 2009) did not differ between treatments. Mating partners were chosen at random without prior screening of microsatellite genotypes. We transferred single non-virgin males from their housing vials to clean vials (28.5 mm diameter X 95 mm tall) and subsequently introduced a single virgin female, observing the pair to ensure that copulation occurred and noting its duration. After copulation had terminated, the female and the male were immediately separated. In a few cases (14 out of 210 copulations), very long copulations were interrupted after 75 minutes. If females belonged to one of the two treatments with oviposition, immediately after mating they were provided with a smear of dung into which they could lay their eggs. Hence, in one treatment oviposition took place on the first day after the first copulation, and in the other treatment oviposition occurred on the second day. All females and males used in this series of double matings were frozen at -80°C late in the evening of the second day.

For the treatment in which oviposition occurred after the first mating, all offspring were sired by the first male and no paternity testing was necessary. For the treatment in which females were allowed to lay eggs after the second copulation, the resulting eggs could have sired by either male. To determine the paternity of these offspring we transferred all clutches of eggs into 100 ml plastic containers with an excess of previously homogenized and frozen cow dung (> 2 g./ larvae; (Amano 1983)). Clutches were raised in climate chambers at constant 22°C, 60 % relative humidity, and 12 h photoperiod. We checked the containers for emerged adults every day until no individuals emerged for four weeks. Emerged flies were immediately frozen at -80°C for subsequent paternity analyses.

Dissections

We isolated stored ejaculates from previously frozen females that had been dehydrated in ethanol for 24 hours before dissection (Tripet et al. 2001). We carefully removed the posterior portion of the female reproductive tract (including the common oviduct, spermathecal ducts,

spermathecae, accessory glands, and bursa copulatrix) from the rest of the female by grasping the genital valves in forceps and tearing them from the abdomen. We separated the spermathecae together with their ducts from the rest of the reproductive tissue under a quality binocular microscope (Leica MZ-12, Leica Microsystems GmbH, Wetzlar, Germany) and photographed them. We then separated dehydrated, and thus solidified, sperm 'pellets' from female spermathecal tissue using very finely sharpened dissecting tweezers viewed under the same binocular microscope. We took great care to extract the entire ejaculate, and although some sperm may have been lost, this quantity relative to the extracted sperm mass is likely to be trivial. Each sperm pellet was transferred separately to a buffer solution (ATL buffer from the QIAamp® DNA Micro Kit, Qiagen; see below). The three sperm pellets from each female, which each originated from a different spermatheca, were amplified and analysed separately to study the skew in sperm storage across spermathecae. In our analyses we distinguish the singlet spermatheca (regardless of the side of the body on which it is found) from the middle and outer doublet spermathecae.

We measured spermathecal area, spermathecal duct length, and hind tibia length as an index of body size using ImageJ software (http://rsbweb.nih.gov/ij/).

Extraction, amplification and analysis of DNA

We used a Chelex extraction method to extract DNA from the heads of all flies. Cropped heads were transferred into 96-well PCR plates kept on ice. We then pipetted 100 µl of 6 % Chelex suspension (Chelex 100®, Na^+-form, particle size 50 – 100 mesh, Fluka) into each well using wide-ended tips. Afterwards we covered the plate with a plastic mat, carefully shook it, and spun down the heads to ensure that the sample was covered in liquid. We used a thermocycler to incubate plates for 60 minutes at 55°C, boil for 9 minutes at 100°C, and then cool down to 20°C. After taking samples out of the thermocycler we again shook them carefully and spun them down, before the plate was stored at 4°C for 10 to 20 hours, and afterwards frozen at -20°C for at least 24 hours before DNA extractions were used for subsequent processing.

We used a special kit designed for use with forensic amounts of DNA sample (QIAamp® DNA Micro Kit, Qiagen AG, Switzerland) to extract the potentially very low number of DNA copies from sperm pellets. We followed the recommended protocols, including adding carrier RNA to buffer AL (1 µl dissolved carrier RNA in 200 µl buffer AL), and the minimum recommended amount of elution buffer AE (20 µl) when extracting DNA from sperm pellets

in order to retain the highest possible concentration of DNA. We then used the QIAGEN® Multiplex PCR Kit to simultaneously amplify four microsatellite loci: SsCa17, SsCa24, SsCa26, and SsCa30 (Demont et al. 2008; Garner et al. 2000). Total PCR reaction volume for the heads was 6 µl: 1µl DNA template, 3 µl QIAGEN Multiplex PCR Master Mix, 1.4 µl distilled water and 0.6 µl microsatellite primer mix (100 µM). Total PCR reaction volume for the sperm was 30 µl retaining the mixing ratio from the heads (e.g. DNA template and all other volumes five times higher than for the heads; note that the total PCR reaction volume for the sperm was only 24 µl in Bussière *et al.* 2009). Cycling conditions for the heads were as follows: 95°C for 15 min, then 27 cycles of 94°C for 30 s, 60°C for 3 min and 72°C for 45 s, and finally 60°C for 30 min. Cycling conditions for the sperm DNA were the same but were run for 30 cycles instead of 27 to allow for the lower initial template concentration. Using these conditions, large stutter bands are usually not produced (Bussière et al, 2009). PCR products were separated on a capillary sequencer (Applied Biosystems 3730 DNA Analyzer), and the output analysed using Applied Biosystems GeneMapper® software. Of the four loci, one (SsCa17) was not sufficiently polymorphic in our samples to adequately correct using the procedure described below, so for the remainder of the paper we shall focus on the other three loci (cf. Bussière *et al.* 2009)

Statistical analyses

We calculated Pearson correlation coefficients (r) between female hind tibia length (a proxy for overall female size), spermathecal duct length and square root spermatheca area, as well as for the dimensions of the reproductive tract among themselves. In addition, we estimated the partial correlation between the organs of the reproductive tract when controlling for hind tibia length. Pearson and partial correlations were computed using SPSS 16 (Anonymous, 2007). Hind tibia length was measured without being stored in 70 % ethanol, but spermathecal duct length and spermathecal area were measured after samples had been dehydrated in 70 % ethanol for 36 hours.

The response variable in all our statistical analyses was S2 (the proportion of stored sperm assigned to the second of two copulating males) estimated by the relative signal intensity of a second male's alleles in the amplified sample of DNA extracted from the sperm pellet (cf. Bussière et al. 2009). Since many factors besides the initial concentrations of alleles (e.g., allele length) may contribute to the observed signal strength of a particular allele after PCR

(Haberl & Tautz 1999), we had to correct measures of relative peak intensity for each of our microsatellite loci to obtain S2 values. A detailed description of how to correct estimates of signal strength is given in Bussière *et al.* (2009).

We performed statistical modelling as recommended in the R Book (Crawley 2007): we started with a maximal model that included all factors, covariates, interactions, and quadratic terms that could be of interest and simplified it in a stepwise manner on the basis of deletion tests (e.g. testing simpler nested models against more complex models: likelihood ratio tests). In contrast to Crawley (2007), who recommends deletion of all explanatory variables (e.g. including main effects) which do not significantly improve the fit of the model, we retained all main effects in the final model even if they did not significantly improve the fit of the model. Therefore, our final model is a kind of "minimal adequate model" in terms of interactions and higher order terms, but with all initial main effects still included, so that readers can better assess the non-significance of certain main effects. All statistical modelling was performed in R 2.6.2 (R Development Core Team, 2008). We analysed the within female skew in sperm storage across the spermathecae and the effect of oviposition on it (i.e., the whole data set with three treatments) using linear mixed effects models fitted with the *lme* function from the *nlme* package (Pinheiro *et al.* 2008), and corrected and arcsine square root transformed S2 values as the response. The maximal model included as explanatory variables treatment, spermatheca identity, female size, size of the two males, spermathecal duct length, square root spermatheca area, both copula durations, all two way interactions, and all quadratic terms.

For the subsample of data for which we had estimates of P2 (i.e. the treatment in which oviposition followed the second copulation), we additionally investigated the relationship between P2 and S2 using linear mixed effects models. We performed statistical modelling with corrected and transformed S2 (and *not* P2) values as the response for two reasons. First, the chronological processing of the flies demanded this: they first laid the eggs and were afterwards frozen and dissected. Consequently, the S2 estimates in the present study describe what is left over in the spermathecae after females laid eggs. Second, fitting mixed models in R 2.6.2 (R Development Core Team, 2008) with P2 as the response would create a system that is computationally singular (e.g. all nine P2 values for one female [3 spermathecae x 3 loci that provided an estimate] had the same value). The maximal model included as explanatory variables spermatheca identity, P2 (arcsine square root transformed), female size, size of the two males, spermathecal duct length, square root spermatheca area, both copula durations, all two way interactions, and all quadratic terms.

All mixed models (for the whole data set and the subsample of data for which we had P2) included the locus that provided a given S2 estimate nested within the spermatheca nested within the female as random effects. Models were fitted using maximum likelihood (ML) during the process of model simplification, while the final models were fitted using restricted maximum likelihood (REML).

Results

We attempted to mate 105 females to two males each (35 for each mating-oviposition treatment). In the treatment without oviposition, one female escaped, two died during the experiment, two failed to mate twice, one had four spermathecae with four ducts, and in one female all three spermathecae were empty, resulting in a sample of 28 females. In the treatment with oviposition between first and second copula, one female died, two failed to mate twice, one had four spermathecae with four ducts, and ten females did not lay eggs, resulting in a sample size of 21 females. In the treatment with oviposition after the second copulation, one female failed to mate twice, one had four spermathecae with four ducts, one had completely empty spermathecae, the spermathecae of one female got lost during dissections, and four females did not lay eggs, resulting in a sample size of 27 females. From these remaining 76 (= 28 + 21 + 27) females, 15 females exhibited a missing value for one measured variable: four females had one spermatheca that was empty (in two cases the singlet spermatheca and in one case each the middle doublet and outer doublet spermatheca), one single spermatheca was lost during dissection, and three spermathecae provided ambiguous results after PCR. For five females we could not measure spermathecal duct length or spermathecal area, and for two females we had one copula duration missing. Final sample sizes for both mixed model analyses can be extracted from Table 2 and 3.

Mean (± SE) spermathecal duct lengths were 662.32 ± 12.61 µm (n = 66 females), 675.20 ± 13.28 µm (n = 70), and 680.25 ± 11.52 µm (n = 67) for the singlet spermatheca, the middle doublet and outer doublet spermatheca, respectively. Mean (± SE) spermathecal sizes (i.e. square root spermathecal areas) were 113.53 ± 1.74 µm (n = 66), 114.61 ± 1.03 µm (n = 71), and 115.45 ± 1.22 µm (n = 68) for the singlet spermatheca, the middle doublet and the outer doublet spermatheca, respectively.

Pearson correlation coefficients (r) were significant among all spermathecal ducts, between all spermathecal sizes, and between female size (hind tibia length) and middle doublet and outer doublet spermathecal size (Table 1). The Pearson correlation between female size and spermathecal size of the singlet spermatheca was marginally non-significant ($p = 0.07$; Table 1). Female size and spermathecal duct lengths, as well as spermathecal duct lengths and spermathecal sizes were all uncorrelated (Table 1). Partial correlations when controlling for female size were significant among spermathecal ducts themselves, and among spermathecal sizes themselves (Table 1). Again, spermathecal duct lengths and spermatheca sizes were uncorrelated (Table 1).

Residuals of both final mixed models were normally distributed (Shapiro-Wilk normality test: $p = 0.06$, whole data set; $p = 0.56$, data set with S2 and P2). The summary of the linear mixed model for the proportion of second male sperm in storage (cf. Bussière *et al.* 2009) for the whole data set is given in Table 2. Treatment, spermathecal identity, spermathecal size, both copula durations, and the interaction term between spermathecal identity and spermathecal duct length significantly influenced S2 (Table 2). The significant effect of treatment indicates that S2 values were highest in the treatment without oviposition and lowest when females laid eggs after the second copula (i.e. used sperm from the second male) (Table 2; Fig. 1). Mean S2 (averaged over all spermathecae and loci, ± SE) were 0.692 ± 0.064, 0.598 ± 0.064, and 0.791 ± 0.042 for the treatment with oviposition after the first copula, after the second copula, and for the treatment without oviposition, respectively. The significant effect of spermatheca indicates a consistently lower S2 estimate for the doublet spermathecae compared to the singlet (Fig. 1). S2 significantly decreased with increasing size of the spermathecae (Fig. 2). First male copula duration had a significant negative effect on S2 (Fig. 3), while the duration of the second male's copula had a significant positive effect on S2 (Fig. 4). The significant interaction between spermathecal identity and the respective length of the spermathecal duct, showed that the influence of the spermathecal duct length on S2 varied across spermathecae: in the singlet spermatheca S2 increased with increasing duct length, in the middle doublet spermatheca S2 decreased with increasing duct length, while in the outer doublet spermatheca duct length did not influence S2 (Fig. 5). Female size and the size of the two males did not significantly affect S2 (Table 2).

The summary of the linear mixed model for the proportion of second male sperm in storage for the subsample of data for which we had sperm storage and paternity data (i.e., the

treatment with oviposition after the second copula) is given in Table 3. Spermathecal identity, second male paternity (P2), spermathecal size, copula duration of the second male, and three two-way interaction terms significantly influenced the skew in sperm storage (S2) among these females (Table 3). S2 values significantly increased in all spermathecae with increasing P2 values (Fig. 6), indicating a strong positive association between paternity success and the proportion of sperm in storage (strictly speaking: the proportion of stored sperm remaining after female fertilized a clutch of eggs). Pearson correlation coefficients (r) between S2 and P2 for the singlet spermatheca, the middle spermatheca and the outer doublet spermatheca were all high, namely 0.902, 0.863, and 0.836, respectively. Mean P2 (± SE) was 0.587 ± 0.073 and matched mean S2 for the corresponding flies (0.598 ± 0.063). The significant effect of spermatheca indicates a consistently lower S2 estimate for the doublet spermathecae compared to the singlet (Fig. 1: treatment with oviposition after the second copula). The significant main effect of spermathecal size and the significant interaction between spermathecal identity and spermathecal size indicate that overall S2 decreased with increasing spermathecal size, but that the magnitude of this decrease differs across the spermathecae (Fig. 7). S2 decreased the most with increasing spermatheca size in the middle doublet spermatheca, while this effect was weakest in the singlet spermatheca (Fig. 7). The significant interaction between P2 and the size of the second male indicated that S2 values increased with P2 as well as with the size of the second male (Table 3). Thus, the highest S2 values were associated with high P2 values and large second males. The significant interaction between spermathecal size and female size highlighted that S2 values decreased with increasing size of the spermatheca as well as with increasing size of the female (Table 3). Therefore, small S2 values were associated with large spermathecae, and this pattern was more pronounced for large females than for small ones. The nature of these last two interactions was detected with interactive 3D scatters and real-time visualization, and is not illustrated here.

Table 1 Correlations between different morphological measurements. Below the diagonal: Pearson correlation coefficients r; above the diagonal: partial correlations controlled for female size.

	Female	Duct 1	Duct 2	Duct 3	Spermatheca 1	Spermatheca 2	Spermatheca 3
Female	1						
Duct 1	0.175	1	**0.721***	**0.772***	0.129	0.004	-0.024
Duct 2	0.029	**0.713***	1	**0.855***	0.007	0.127	0.126
Duct 3	0.006	**0.761***	**0.859***	1	0.083	0.048	0.136
Spermatheca 1	0.222[#]	0.144	0.008	0.068	1	**0.342***	**0.256***
Spermatheca 2	**0.306***	0.052	0.148	0.121	**0.388***	1	**0.571***
Spermatheca 3	**0.385***	0.056	0.125	0.124	**0.342***	**0.595***	1

Female: female size (i.e. hind tibia length); Duct: spermathecal duct length; Spermatheca: square root spermatheca area.

*** Significant at the 0.05, 0.01, or 0.001 level.**

[#] p = 0.07.

Table 2 Summary of the linear mixed model for arcsine square root transformed proportion of second male sperm in storage as a function of oviposition-treatment, the spermatheca and morphological and behavioural covariates. The model included the locus that provided a given estimate nested within the spermatheca nested within the female as random effects.

	Numerator df	Denominator df	F value	p value
Intercept	1	283	818.0930	**<0.0001***
Treatment	2	61	5.5278	**0.0062***
Spermatheca	2	119	6.4518	**0.0022***
Female size	1	61	0.5504	0.4610
Male 1 size	1	61	1.5699	0.2150
Male 2 size	1	61	0.2653	0.6083
Spermathecal duct length	1	119	0.5891	0.4443
Square root spermathecal area	1	119	5.7597	**0.0179***
Male 1 copula duration	1	61	6.1010	**0.0163***
Male 2 copula duration	1	61	11.2180	**0.0014***
(Male 2 copula duration)^2	1	61	19.4944	**<0.0001***
Spermatheca X Spermathecal duct length	2	119	3.2273	**0.0432***
Spermatheca X Male 2 copula duration	2	119	2.7074	0.0708

*** Significant F-tests.**

Table 3 Summary of the linear mixed model for arcsine square root transformed proportion of second male sperm in storage as a function of spermatheca, second male paternity and morphological and behavioural covariates. The model included the locus that provided a given estimate nested within the spermatheca nested within the female as random effects.

	Numerator df	Denominator df	F value	p value
Intercept	1	115	852.5903	**<0.0001***
Spermatheca	2	37	5.3151	**0.0094***
Second male paternity (arcsine square root)	1	18	156.7746	**<0.0001***
Female size	1	18	0.0056	0.9411
Male 1 size	1	18	1.0056	0.3293
Male 2 size	1	18	0.6598	0.4272
Spermathecal duct length	1	37	0.5114	0.4790
Square root spermathecal area	1	37	7.8881	**0.0079***
Male 1 copula duration	1	18	1.6880	0.2103
Male 2 copula duration	1	18	2.9841	0.1012
(Male2 copula duration)^2	1	18	9.1753	**0.0072***
Spermatheca X Spermathecal duct length	2	37	2.5717	0.0900
Spermatheca X Square root spermathecal area	2	37	7.0492	**0.0025***
Second male paternity X Male 2 size	1	18	7.8986	**0.0116***
Female size X Square root spermathecal area	1	37	4.5069	**0.0405***

Second male paternity (the proportion of paternity assigned to the second of two copulating males) was arcsine square root transformed for analyses.

*** Significant F-tests.**

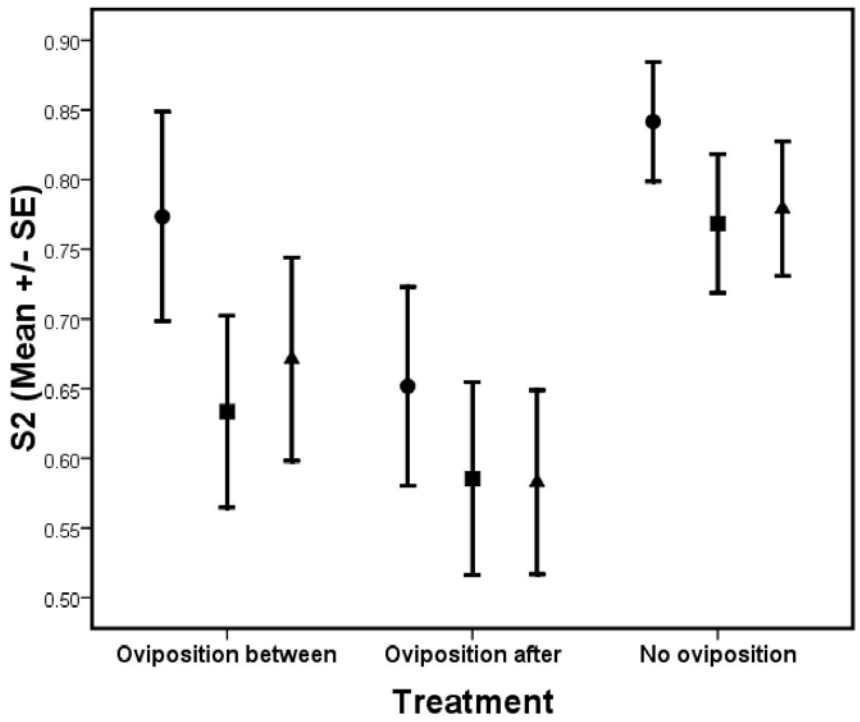

Fig. 1 Mean (± SE) proportion of second male sperm in storage in each of the three spermathecae (circles: singlet spermatheca; squares: middle doublet spermatheca; triangles: outer doublet spermatheca) as a function of oviposition treatment.

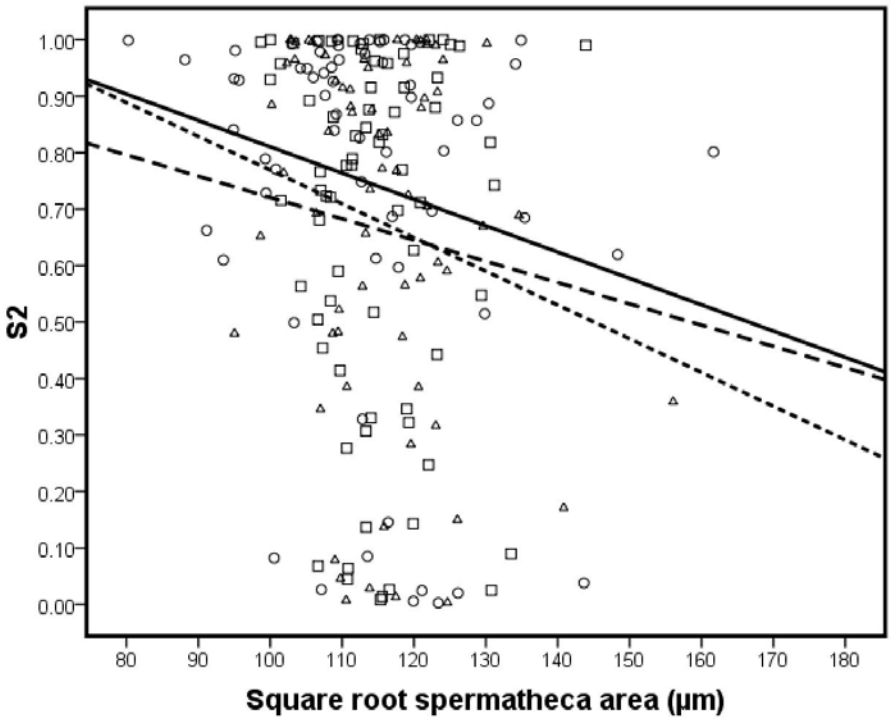

Fig. 2 Proportion of second male sperm in storage in each of the three spermathecae (solid line: singlet spermatheca; dashed line: middle doublet spermatheca; dotted line: outer doublet spermatheca) as a function of square root spermathecal area.

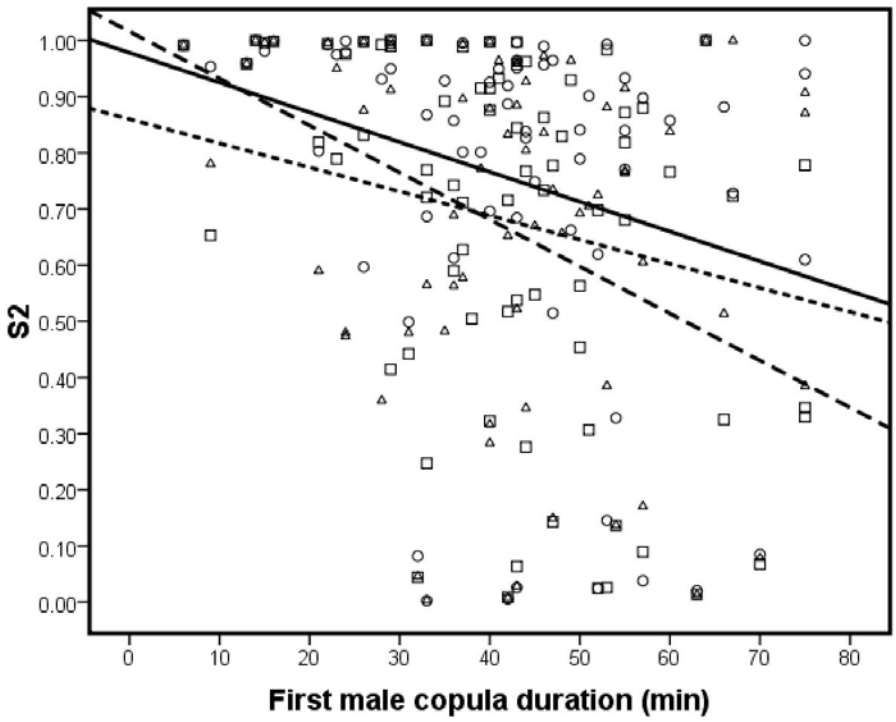

Fig. 3 Proportion of second male sperm in storage in each of the three spermathecae (solid line: singlet spermatheca; dashed line: middle doublet spermatheca; dotted line: outer doublet spermatheca) as a function of first male copula duration.

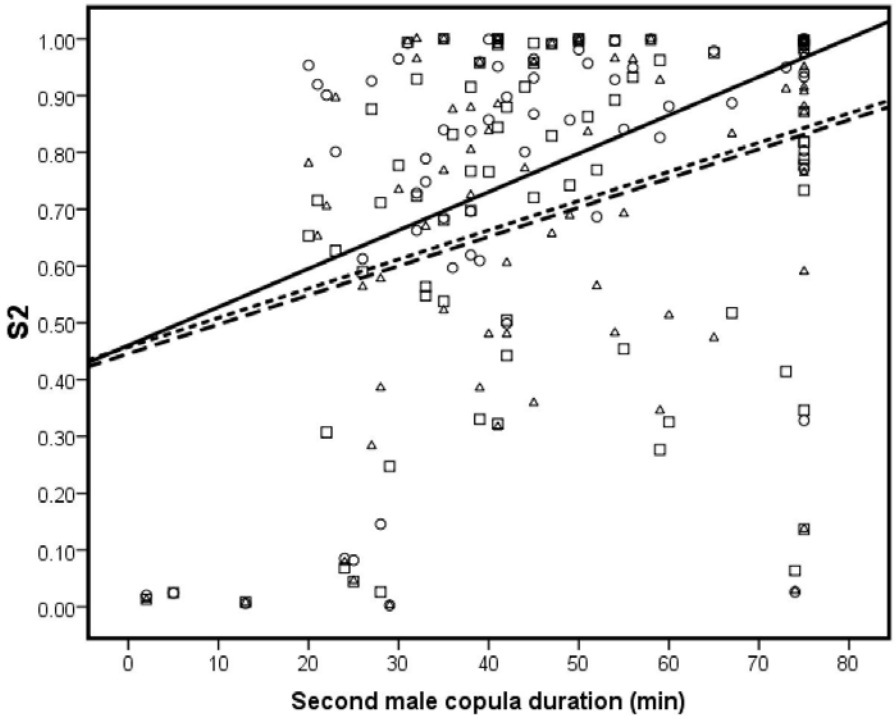

Fig. 4 Proportion of second male sperm in storage in each of the three spermathecae (solid line: singlet spermatheca; dashed line: middle doublet spermatheca; dotted line: outer doublet spermatheca) as a function of second male copula duration.

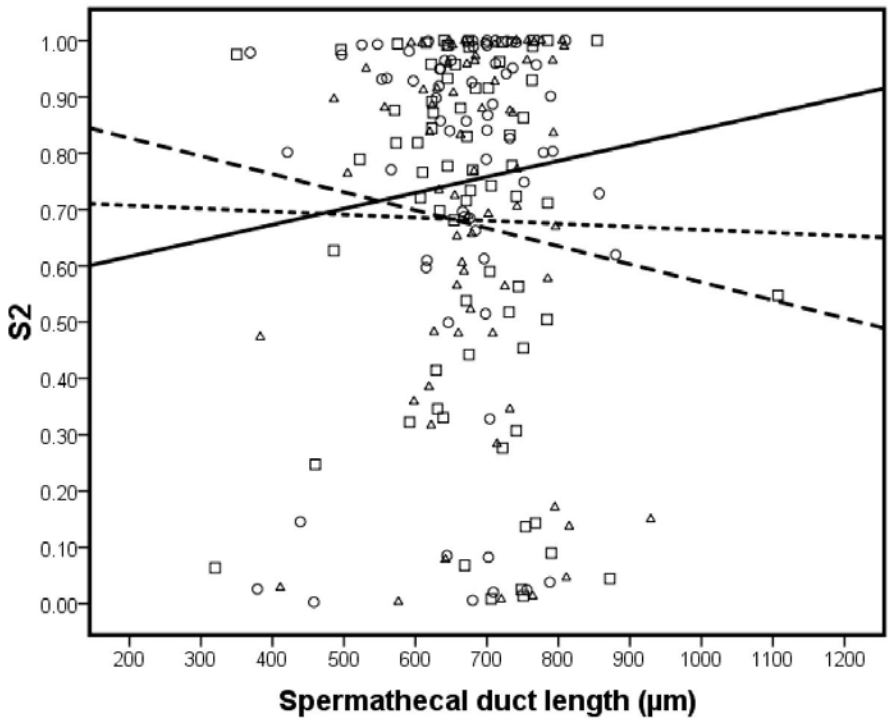

Fig. 5 Proportion of second male sperm in storage in each of the three spermathecae (solid line: singlet spermatheca; dashed line: middle doublet spermatheca; dotted line: outer doublet spermatheca) as a function of spermathecal duct length.

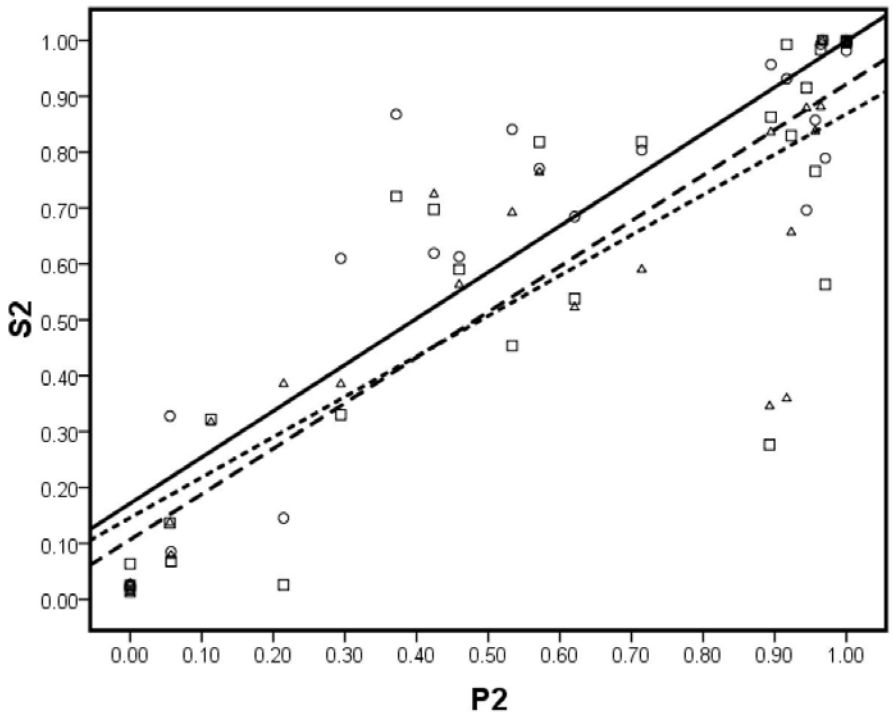

Fig.6 Proportion of second male sperm in storage (remaining after oviposition) in each of the three spermathecae (solid line: singlet spermatheca; dashed line: middle doublet spermatheca; dotted line: outer doublet spermatheca) as a function of the proportion of second male paternity.

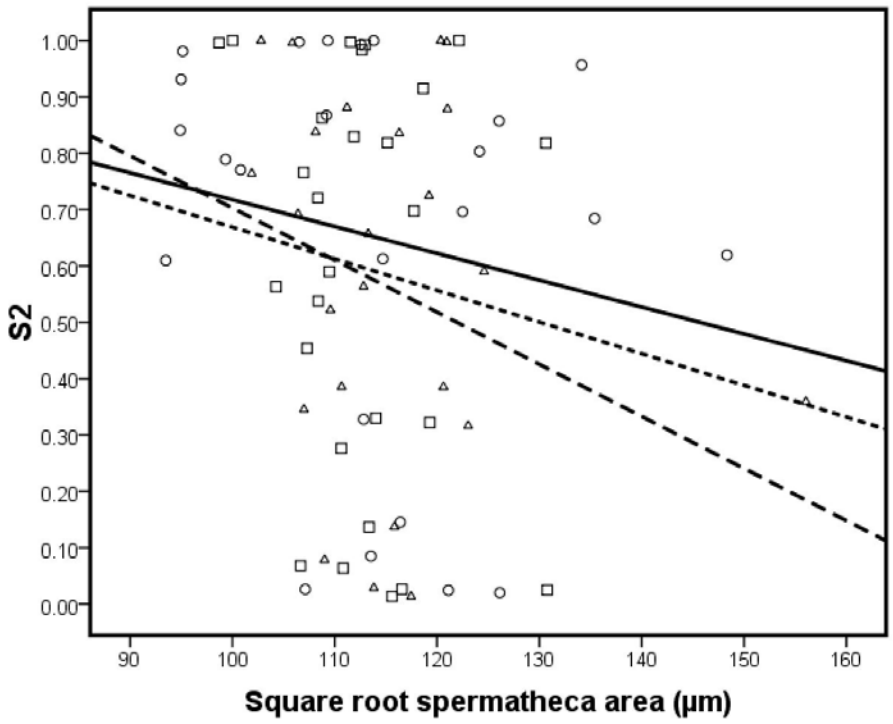

Fig. 7 Proportion of second male sperm in storage in each of the three spermathecae (solid line: singlet spermatheca; dashed line: middle doublet spermatheca; dotted line: outer doublet spermatheca) as a function of square root spermathecal area.

Discussion

Clarifying the mechanics of sperm transfer and use within females is crucial for assessing the relative contributions of male and female influences on differential fertilization success (Bussiere et al. 2009; Eberhard 1996; Luck et al. 2007; Pai & Bernasconi 2008; Simmons 2001). Traditionally, cryptic female choice has received less attention than sperm competition in spite of its potentially strong influence because of practical constraints on investigating sperm transfer (Eberhard 1996; Eberhard 1997; Simmons 2001). However, recent advances in direct anatomical investigation (Arthur et al. 2008; Hosken et al. 1999; Sbilordo et al. 2009) combined with newly developed molecular techniques (Bussiere et al. 2009; Hall et al. 2010) promise to clarify the events mediating sperm transfer and use.

In the present study, we used competitive microsatellite PCR to study sperm storage in yellow dung flies and obtained four main results. First, oviposition strongly influenced sperm storage patterns, but the pattern of storage was not as we had predicted using simplistic assumptions about how sperm usage affects the remaining sperm in storage. Second, we confirmed a

previously reported consistent skew in storage across spermathecae (Bussiere et al. 2009), with more second male sperm stored in the singlet than in either doublet spermatheca. Third, morphological (e.g. spermatheca size) and behavioural (e.g. copula duration) covariates and several two-way interactions significantly influenced S2, indicating a complex interplay of female and male influences on sperm storage. Fourth, S2 and P2 values were strongly correlated, implying that paternity is largely assigned in proportion of the amount of stored sperm, although our data do not exclude subtle female influences during sperm utilization at fertilization (i.e. sperm selection). Below, we first discuss findings for the whole data set (three oviposition-treatments), and then we focus on the subsample of data for which both sperm storage and paternity data are available.

Sperm storage across all three oviposition-treatments and associated postcopulatory processes

The presence and timing of oviposition relative to mating significantly influenced S2. Before executing the experiment, we made predictions based on how sperm usage during fertilization ought to influence the relative numbers of sperm in storage. Our experiment indicates that these expectations were simplistic because they failed to take account of the complexity of sperm transport and possible adaptive changes by males in response to the gravid status of a female (Wedell et al. 2002).

In yellow dung flies, average P2 is often reported at about 80 % (Bussiere et al. 2009; Parker 1970d), and remains constant in successive clutches without additional matings (Parker 1970d). Our mean proportion of last male sperm in storage (over all spermathecae and loci, ± SE) for flies that did not lay eggs was 79.1 ± 4.2 %, which is consistent with these previous estimates. However S2 for flies that oviposited after the second copula was significantly lower, at 59.8 ± 6.3 %, indicating one of two phenomena: either disproportional more sperm from the second male was utilized to produce the clutch of eggs, or the act of laying eggs itself interrupted complete sperm transfer and displacement (e.g., because the first eggs descending from the common oviduct push out most of the sperm remaining in the bursa copulatrix and thereby prevent the post-copulatory transfer of these sperm from the bursa to the spermathecae). These alternatives predict different paternity outcomes: in the first situation, second male paternity should be higher than 59.8 % (i.e. mean S2), whereas it need not be different from 59.8 % in the second situation. Mean (± SE) second male paternity was 58.7 ± 7.3 %, clearly favouring the later explanation.

Mean S2 and P2 were with 59.8 % and 58.7 % respectively, lower than previous reported for similar situations when mating was immediately followed by oviposition (e.g. Chapter 5). At the moment we can only speculate what caused the differences between mean P2 values of different studies. Usually males guard females after terminated copula. If this behaviour would somehow assist sperm transfer to the spermathecae and subsequent utilization, some of the variance in reported mean P2 values could be attributed to the fact that some studies allowed mate guarding during oviposition and others not (e.g. this study vs. Chapter 5). However, other environmental or laboratory sources that affect mean P2 can not be excluded.

Interestingly, flies without oviposition also showed higher proportions of second male sperm than flies which oviposited after the first copula (i.e. used sperm from the first male: 69.2 ± 6.4 %). If the only differences between these treatments had been the number of sperm used for fertilizing the first clutch, the opposite pattern, with higher S2 in the treatment with oviposition, would be expected. However male dung flies are known to adjust investment in copula in response to females including the number of eggs carried by them (Parker et al. 1999). In nature, females who do not carry a mature clutch are a much less valuable resource to males because females almost invariably mate immediately prior to egg laying. A male mating a female without a full clutch is therefore likely to have his ejaculate supplanted just prior to oviposition, and therefore is expected to invest less in such a copulation. In accordance with this expectation (Parker 1970c; Parker 1970d; Parker et al. 1999), we found that that the second males in the treatment featuring oviposition between matings copulated for shorter durations than those in other treatments (linear model with second male copula duration as response: $F_{2,71} = 4.027$, $p = 0.02$). The difference in sperm storage pattern can therefore be accounted for by this shift in male allocation (Parker 1970c; Parker 1970d; Parker et al. 1999; Wedell et al. 2002), and whatever the difference in storage that arises due to depletion of first male sperm is swamped by plasticity in copula durations which is apparently largely controlled by males (Parker 1970c; Parker 1970d; Parker & Simmons 1994).

Our study further revealed consistent skew in sperm storage across spermathecae, with more last male sperm stored in the singlet than in either doublet spermatheca. The pattern was present in all treatments, and matches the pattern identified previously in an experiment that manipulated mating interval to study its influence on sperm storage (Bussiere et al. 2009). The consistently highest level of sperm displacement associated with the singlet could explain

why field-caught female yellow dung flies have sperm from fewer males in the singlet spermatheca than in either doublet spermatheca (Chapter 4 and 5). Whether this pattern is a consequence of male or female effects cannot be directly assessed here, but we think that the requirement for active female musculature for effective sperm transfer makes a females influence more likely, since males do not penetrate the spermathecal ducts with their intromittent organs. It is also unlikely that a consistent order of filling for the spermathecae can explain this pattern (e.g., if the singlet is always the first spermatheca to be filled), because such a scenario would predict an interaction between second male's copula duration and the strength of difference in storage across organs, which both the present data and an earlier study do not support (Bussiere et al. 2009). Although we cannot determine whether skew across spermathecae is adaptive from the current work, such a pattern is a prerequisite for adaptive sperm selection (Bussiere et al. 2009; Hellriegel & Bernasconi 2000; Hellriegel & Ward 1998; Ward 2000).

Copula duration of both males significantly influenced the amount of stored sperm from the second male. Several studies have already documented the significant influence of copula duration on the number of transferred sperm or paternity success (Bussiere et al. 2009; Parker & Simmons 1991; Parker & Simmons 1994; Parker & Simmons 2000; Simmons et al. 1999), so we neglect a detailed discussion of these finding here. In addition to these behavioural covariates, female reproductive tract dimensions also influenced S2, which also accords with other work (Wuest & Bussière, in preparation; Wuest et al., unpublished). S2 values significantly decreased with increasing size of the spermathecae, indicating that sperm displacement is weaker in large spermathecae. Spermathecal volume correlates with female body size (this study; (Parker et al. 1999), and males appear to compensate for the decreased sperm displacement rate in large females by copulating longer with larger females (Parker et al. 1999). S2 was also significantly influenced by the length of the spermathecal duct, although the effect of duct length differed across the three spermathecae. In the singlet, S2 increased with increasing duct length, suggesting that long singlet ducts facilitate sperm displacement (e.g. through increased muscular force and thus higher sperm transfer to the spermatheca). This pattern was reversed in the middle doublet spermatheca, and absent for the outer doublet spermatheca; a compelling explanation for this difference across spermathecae is elusive.

Relationship between S2 and P2 and associated postcopulatory processes

Only females that oviposited after the second copula produced clutches with potentially mixed paternity broods, and so our investigation of how patterns of sperm storage (S2) and paternity (P2) covary is therefore restricted to them. Overall, S2 and P2 values were strongly correlated, but the strongest correlation with P2 was for estimates of S2 within the singlet spermatheca ($r = 0.902$). The doublet spermathecae featured slightly weaker correlations with P2 (middle doublet $r = 0.863$; outer doublet $r = 0.836$). These findings are consistent with the singlet being the preferred organ of storage and fertilization, as has been reported for a dryomyzid fly (Otronen 1997), but which has been inconsistently demonstrated in yellow dung flies (Hellriegel & Bernasconi 2000; Otronen et al. 1997; Ward 1993; Ward 1998). The magnitude of the correlations further suggests that once sperm are stored within spermathecae, they are largely used according to their numerical representation (i.e., in an "ideal lottery"). This does not imply that females exert no influence during sperm utilization (e.g. sperm selection), since still some variance in the relationship between S2 and P2 is unexplained. We also note that our estimates of S2 may not perfectly represent the sperm mixture available prior to oviposition since we could not sample the spermathecal contents until after egg laying had completed for obvious reasons.

The significant interaction between P2 and size of the second male, and between spermatheca size and female size indicate that male and female body sizes both subtly influence postcopulatory processes. The proportion of second male sperm in storage after oviposition was strongly associated with P2. Furthermore, the body size of the second male strengthened this effect: highest S2 values after oviposition were associated with high P2 values especially if males were large. Currently, we cannot distinguish if female (different transfer rates depending on mating partner size) or male (different insemination ability that covaries with body size) account for the observed pattern.

S2 values decreased with increasing spermatheca size, and this effect was especially apparent in large females, indicating that spermathecal volume *and* female body size influenced sperm displacement. Decreased sperm displacement rates for large females have been reported previously, and as noted above males appear to compensate for this by copulating longer with larger females (Parker et al. 1999). However, Parker and colleagues (1999) were unable to detect an influence of spermathecal volume on copula duration in their study.

Our work has clarified several exciting new aspects relating sperm storage to use in yellow dung flies, but the complexities of the mechanics involved preclude simple explanations. The behavioural plasticity that has been demonstrated for both sexes in this species suggests that the outcome of competition for fertilization will never be determined by simple rules, but rather by sometimes subtle and often complex interactions between a female, her multiple mates, the circumstances surrounding the copulations, the intervals between them, the time to oviposition, and the environmental conditions that prevail when oviposition occurs. While we continue to slowly advance our knowledge about the possible mechanisms mediating sperm competition and cryptic female choice, convincing evidence for some of these mechanisms (e.g. sperm selection) is still missing, and the relative contributions of male and female mechanisms during successive postcopulatory stages to differential fertilization success remain partly unexplained. We are convinced that combining direct anatomical observation of processes occurring within females with new methods for quantifying sperm in storage and direct estimates of paternity success will continue to clarify the mechanisms underlying nonrandom paternity in this and many other species of interest.

Acknowledgements

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Chapter 4

Polyandry in the wild: differential sperm storage and temporal changes in sperm competition intensity in yellow dung flies

Marco Demont, Claudia Buser, and Luc F. Bussière

Abstract

Polyandry is omnipresent in insects. Nevertheless, the evolutionary causes and consequences of this phenomenon remain debated. The lack of information about natural mating rates and the fact that most postcopulatory processes are hidden from view within female reproductive tracts strongly contribute to this controversy. We captured wild female yellow dung flies *Scathophaga stercoraria* over the whole spring season and genotyped the sperm from their spermathecae to obtain field information on sperm transfer, sperm storage, and prevalent levels of polyandry for this model species of postcopulatory sexual selection research. On average females stored sperm from 2.47 males based on a minimum estimate, and 3.33 based on a probabilistic estimate that incorporates population allele frequencies, respectively. Sperm storage and therefore sperm competition intensity showed high temporal variation: the proportion of multiply mated females (i.e. females with sperm from ≥ 2 males within their sperm stores) and the absolute number of ejaculates detected within females strongly increased over the spring season before it sharply decreased at the end. Interestingly, we detected a positive relationship between the number of stored ejaculates and females' wing injuries, suggesting a mechanism by which males may be able to assess prevalent sperm competition situation. Our study found no indication of intraejaculate sperm sorting, but importantly, the number of ejaculates in storage differed amongst the three sperm storage organs (spermathecae) of female yellow dung flies. Different sperm mixtures across the spermathecae could enable females to bias paternity towards certain males, if females are capable of selectively using sperm from a certain spermatheca at the time of fertilization. Data from natural populations as in the present study are essential to promote research on polyandry and postcopulatory sexual selection.

Introduction

Polyandry (females mating with more than one male) is nearly ubiquitous in the animal kingdom. Nevertheless, the evolutionary causes and far-reaching consequences of polyandry remain the subject of debate (Arnqvist & Nilsson 2000; Arnqvist & Rowe 2005; Evans & Simmons 2008; Jennions & Petrie 2000; Simmons 2005). This is especially true if there are no obvious direct benefits associated with female remating, for example the replenishing of their sperm stores or the acquisition of food from mating partners. In such cases, repeated mating by females might arise via a number of alternative nonadaptive (e.g. correlated response to sexual selection on multiple mating by males (Halliday & Arnold 1987)) or adaptive mechanisms, including the acquisition of high quality or compatible genes (indirect benefits). The relative importance of each of these alternatives is currently unknown both in general and for many specific examples of female polyandry.

While many important laboratory studies have attempted to clarify the forces acting on female mating rates (Martin & Hosken 2003; Tregenza & Wedell 2002), extrapolating results to the natural situation in wild populations is difficult. Partly responsible for this is the fact, that we often do not know if our laboratory settings really reflect the situation in wild populations (Bretman & Tregenza 2005; Simmons et al. 2007). This lack of information about the natural situation implies a considerable risk to progress in the study of polyandry and its consequences, and at worst could even lead to misinterpretation of data generated in the laboratory. Therefore, better documentation of natural levels of polyandry in wild populations, ideally featuring analyses of spatial and/or temporal variation, are needed. Studies of the ecological and evolutionary factors that alter selection on female remating rates in the field are also of crucial importance (Wilson 2009).

Assessing the degree of polyandry by directly observing mating in the field poses a challenge, especially for small and mobile species such as insects. One approach is to genotype the sperm within the sperm stores of females to assess the number of mates (Chapuisat 1998; Krieger & Keller 2000). Since copulations may not always result in successful sperm transfer and sperm from recent mates may have displaced sperm from previous males, this estimate of female mating frequency (the genetic mating frequency) may underestimate the actual mating frequency in the field (the social mating frequency). Nevertheless, this estimate of mating frequency obtained by genotyping

stored sperm from females is a good measure of the *minimum* degree of polyandry prevalent in the wild, a parameter that is probably more important for male sexual behaviour than the social mating frequency of females (e.g. copulations without sperm transfer and/or for which transferred sperm have since been displaced).

Next to polyandry, is sperm storage the second prerequisite for sexual selection via certain forms of cryptic female choice (e.g. sperm selection) and sperm competition. Therefore, thorough studies of polyandry in wild populations must be coupled with a precise investigation of sperm storage patterns in females. Sperm competition has been shown to be a particularly potent evolutionary force, shaping males' behaviour and anatomy (Parker 1970c; Simmons 2001). Females present the environment where sperm competition takes place, making the interdependence of the two postcopulatory mechanisms of sperm competition and cryptic female choice obvious. Numerous mechanisms of both processes have been identified (Eberhard 1996; Simmons 2001). However, the degree to which females can influence sperm transfer, sperm storage, sperm sorting, and in the last instance choose particular sperm for fertilizing their eggs remains unclear (Birkhead & Pizzari 2002). As a result, the extent and relative importance of sperm competition and cryptic female choice to differential fertilization success are still often unknown (Snook 2005). A better describing and understanding of the processes occurring during copulations and patterns observed within mated females is one necessary step in unravelling the mystery of postcopulatory incidents and their evolutionary consequences, and we not just need this information from laboratory settings, but again also from wild populations.

Varying levels of polyandry do not only affect the *number of ejaculates* that compete within the female for fertilization of the ova, but also *how much ejaculate* from each male is present in the contest (Wedell et al. 2002). Several studies have shown that males adjust their reproductive behaviour according to the risk of sperm competition (indicated by the level of polyandry). For example, when subject to higher risks of sperm competition, elephant seals (*Mirounga angustirostris*) show more aggressive behaviour against rival males (Leboeuf & Peterson 1969), and flour beetles (*Tenebrio molitor*) increase their mate guarding (Gage & Baker 1991). Additionally, males respond at the gametic level to sperm competition risk (Pizzari et al. 2003; Wedell et al. 2002). Comparative studies have consistently shown a positive relationship between the degree of polyandry (an index of sperm competition) and relative testis size (a standard index of investment in sperm) amongst related taxa (Gage 1994; Hosken 1997). Within species, males adjust their ejaculate expenditure during a particular mating event according to cues arising from other

conspecifics (males and females). Several studies provided evidence that increased risk of sperm competition displayed by the presence of rival males resulted in increased ejaculate size (Gage 1991; Pound & Gage 2004). Males also strategically allocate their sperm according to female mating status and/or quality (Martin & Hosken 2002; Wedell 1998). Exactly how males detect female mating status (e.g. virgin vs. mated) and/or the number of sperm or ejaculates stored by females is often unclear (Engqvist 2007) [but see (Carazo et al. 2004; Thomas & Simmons 2009)]. Importantly, the relationship between sperm competition risk (the probability that a female will mate with more than one male) and sperm competition intensity (the number of males involved in sperm competition) is not always straightforward (Engqvist & Reinhold 2005). For example, there may be few males present at mating sites (i.e. low sperm competition risk), but females might have already mated several times and stored sperm from several males (i.e. high sperm competition intensity). This example illustrates that cues arising from other males (e.g. operational sex ratio) and cues arising from the female (e.g. female mating status) may affect males very differently. Just like research on polyandry in general, empirical research on strategic sperm allocation (a consequence of varying levels of polyandry) suffers a bias towards laboratory studies. Data from wild populations that directly assess the number of males involved in sperm competition are needed to help test predictions derived from theoretical models on the evolution of male sperm expenditure.

Yellow dung flies are found throughout the northern hemisphere (Parker 1970a). They overwinter as pupae in the soil or dung pat and emerge as adults in early spring (Blanckenhorn 1998). Phenologies differ at different altitudes and different latitudes (Blanckenhorn 1998; Blanckenhorn & Demont 2004). In the lowlands of Switzerland (e.g. at our study site), adult flies are typically present from late March or early April to mid June and from early September to mid November (Blanckenhorn 1998). It is assumed that flies spend the summer in wooded and cooler areas close to the pastures in a stage of reproductive quiescence (Blanckenhorn et al. 2001). The spring and autumn seasons each consist at most of two generations (Blanckenhorn 1998). Taking into account temperature-dependent development and sexual maturation, sexual mature dung flies of the second spring generation are expected on the pasture in about the middle of May (Blanckenhorn et al. 2001). A study from the same Swiss population featured in the present study has confirmed this time for the appearance of sexually mature dung flies of the second generation on and around dung pats: total wing injuries of dung flies (estimated by a combined measure of tears, notches, and large missing areas) show a seasonal pattern with a springtime peak in mid May (Burkhard et al. 2002).
Copulations usually take place directly on dung pats or in the grass nearby. Directly after copulation, females lay their eggs on the dung, where the larvae hatch and develop. During

oviposition, the male guards the female to prevent copulation with other males. The operational sex ratio is male biased and there is strong precopulatory male-male competition. Several studies have detected mating advantages for large males. A comprehensive study by Jann and colleagues (2000) revealed that in two consecutive years, the number of males per dung pat decreased, while the operational sex ratio increased over the spring season, i.e. more females per male were available in late spring. In addition, the intensity of sexual selection intensity favouring large males was strong in both years (0.499 ± 0.053 and 0.510 ± 0.051), and as expected increased with male density, and therefore decreased over the spring season (Jann et al. 2000).

Male-male competition seems to drive precopulatory sexual selection in *S. stercoraria*, and several studies reported a large impact of males on postcopulatory processes and resulting fertilization (e.g. copula duration and male size, that are both correlated with the amount of sperm transferred (Parker & Simmons 1994; Parker & Simmons 2000)). Nevertheless, theoretical models and data support a significant female influence on sperm storage, displacement and utilization. In particular, the complex reproductive morphology of females is thought to have evolved to facilitate sperm choice. This morphology features a single spermatheca on one side of the body (called the singlet), and two spermathecae on the other side (known as the doublet), each supplied with its own duct. There is some evidence for sperm selection based on male PGM genotype, and females selected for higher numbers of spermathecae (occasionally wild females have four instead of the usual three sperm storage organs) have lower last-male sperm precedence than females selected for only three spermathecae (Ward 2000). Recent work using microsatellite competitive PCR clearly demonstrated that the proportion of rival males sperm do differ between spermathecae (Bussière et al. 2009). Such differences in sperm storage across the spermathecae could provide females with a mechanism to bias paternity towards certain males, provided that females have the ability to differentially use sperm from the three spermathecae for fertilization.

The present study was conducted to gather indispensable information on sperm storage patterns and natural levels of polyandry in yellow dung flies. In particular, we captured wild yellow dung fly females over the whole spring season and genotyped the sperm from their spermathecae to address the following questions: i) How many ejaculates compete within the sperm storage organs of wild flies?; ii) Do patterns of sperm storage by females show temporal variation? Specifically, we asked if the proportion of multiply mated females (i.e. females with two or more males detected within their sperm stores) changes over the spring season, and if the absolute number of ejaculates detected within the spermathecae change over time; iii) Does intraejaculate sperm sorting occur in female

yellow dung flies?; iv) Does the number of ejaculates in storage differ amongst spermathecae?; and v) Does female phenotype (e.g. size, wing injuries) covary with sperm storage?

Materials and Methods

Field sampling

We studied a dung fly population on a pasture in Fehraltorf, near Zurich, Switzerland (8.55°E, 47.37°N). The sampling period covered virtually the complete spring season 2006. We sampled a total of 92 females, each associated with a copulating male, on the following sample dates: 7 copulating pairs on 24 April; 22 pairs on 25 April; 17 pairs on 20 May; 13 pairs on 25 May; 12 pairs on 14 June; 6 pairs on 16 June; and 15 pairs on 22 June. We ascertained that females were copulating by ensuring that genital contact was occurring (in dung flies, males engage in prolonged post-copulatory guarding while positioned over females during the so-called "passive phase"; (Parker 1970b)). If pairs were still copulating 15 minutes after catching them and enclosing them in a vial, the male and female were separated to avoid unnatural extended copulations in the absence of precopulatory male-male competition (e.g. take-overs) and resulting complete displacement of previous stored sperm. Our sample thus involves females that all copulated ≥ 15 minutes with the last male. We transported all individuals to the laboratory where they were immediately frozen at -80°C for later dissection, measurement, and microsatellite genotyping.

Dissections and morphological measurements

We extracted sperm from the spermathecae using a method originally developed to investigate gene flow between *Anopheles gambiae* populations (Tripet et al. 2001). First, we separated the abdomen of the dung fly females from the rest of the body. Head and thorax together with legs and wings were immediately refrozen at -80°C for subsequent processing (see below). In contrast, the abdomen were stored for 48 hours in 70% ethanol (Tripet et al. 2001). Under a quality binocular microscope (Leica MZ-12, Leica Microsystems GmbH, Wetzlar, Germany) we afterwards carefully removed the posterior part of the female reproductive tract (including the common oviduct, spermathecae, spermathecal ducts, accessory glands, and the bursa copulatrix) from the rest of the female's abdomen by grasping the genital valves with forceps and tearing them from the abdomen. Next, we separated the three spermathecae individually from the rest of the reproductive system and

transferred them individually to a drop of water. For every female, we could easily distinguish the singlet spermatheca (regardless of the side of the body on which it is found) from the middle and outer doublet spermathecae (Hosken et al. 1999). We removed all tissue that surrounded the spermatheca and then applied soft pressure to the spermathecal capsule. In this way, we carefully broke the spermatheca open, and since the storage in 70% ethanol caused the ejaculate in the spermatheca to coagulate we were able to take out a sperm pellet from every single spermatheca (cf. Bussière *et al.* 2009). The three sperm pellets from each female, which each originated from a different spermatheca, were transferred to 180 µl of buffer solution (ATL buffer from the QIAamp® DNA Micro Kit, Qiagen; see below) and immediately stored at -80°C for subsequent DNA extraction.

From the rest of the females' body (never stored in ethanol) and the males we assessed several morphological characteristics. We measured left or right hind tibia length of all animals as an index of body size. In addition we counted female left wing injuries according to Burkhard and colleagues (2002). All wing injuries were classified into one of the following three categories: tears, notches, and large missing areas (for a detailed description of this classification see (Burkhard et al. 2002). Burkhard *et al.* (2002) suggested (but did not explicitly test) that tears and notches, i.e., small wing injuries, reflect regular wear, while large missing areas reflect intra- and/or inter-specific interactions. We only measured wing injuries of the left wing, because on average both wings are injured equally: the number wing injuries (tears, notches, and large missing areas) does not differ between left and right wings in yellow dung flies (Burkhard et al. 2002).

Extraction, amplification and analysis of DNA

We performed DNA extraction from sperm pellets according to Bussière *et al.* (2009): we used a kit designed for small amounts of DNA sample (QIAamp® DNA Micro Kit, Qiagen AG, Switzerland) to extract the potentially very low number of DNA copies from sperm pellets. We added carrier RNA to buffer AL (1 µl dissolved carrier RNA in 200 µl buffer AL), and we used the minimum recommended amount of elution buffer AE (20 µl) to retain the highest possible concentration of sperm DNA. As described in Bussière *et al.* (2009), we used the QIAGEN® Multiplex PCR Kit to simultaneously amplify four microsatellite loci: SsCa17, SsCa24, SsCa26 (Garner et al. 2000), and SsCa30 (Demont et al. 2008). Total PCR reaction volume for the sperm pellets was 30 µl (cf. Bussière *et al.* 2009 used only 24 µl): 5 µl DNA template, 15 µl QIAGEN Multiplex PCR Master Mix, 7 µl distilled water and 3 µl microsatellite primer mix (100 µM). Cycling conditions for the

sperm were as follows: 95°C for 15 min, then 30 cycles of 94°C for 30 s, 60°C for 3 min and 72°C for 45 s, and finally 60°C for 30 min. These cycling conditions did not produce large stutter peaks for any of the four applied markers.

We used a Chelex extraction method to extract DNA from the heads of all flies. To our knowledge this method has never before been applied in yellow dung flies, so we describe it here in detail. Cropped heads were transferred into 96-well PCR plates kept on ice. We then pipetted 100 µl of 6 % Chelex suspension (Chelex 100®, Na^+-form, particle size 50 – 100 mesh, Fluka) into each well using wide-ended tips. Afterwards we covered the plate with a plastic mat, carefully shook it, and spun down the heads to ensure that the sample was covered in liquid. We used a thermocycler to incubate plates 60 minutes at 55°C, boil for 9 minutes at 100°C, and then cool down to 20°C. After taking samples out of the thermocycler we again shook them carefully and spun them down, before the plate was stored at 4°C for 10 to 20 hours, and afterwards frozen at -20°C for at least 24 hours before DNA extractions were used for subsequent processing. DNA template amount (1µl), total PCR reaction volume (6µl), and cycling parameters (number of cycles: 27) for the heads were the same as in Bussière *et al.* 2009.

All PCR products from sperm and heads were separated on a capillary sequencer (Applied Biosystems 3730 DNA Analyzer), and the output analysed using Applied Biosystems GeneMapper® software. Head genotypes were simple to score. Sperm samples were more challenging because of the number of alleles present. To avoid artificial inflation of our estimate of the number of males present in the sperm stores, we did not consider very small peaks on either side of a large peak since they potentially represent stutter peaks. In the paragraphs below we describe two different procedures to estimate the number of males detected in each spermatheca.

First, the most conservative method counts alleles and divides by two: to do so we first checked for maternal contamination, and when maternal alleles were present in the allele array, we discounted them. We then identified the alleles from the last male in the array and subtracted those from the total. We then divided the remaining alleles by two, because every male could potentially be heterozygous, rounding up when there were an odd number of remaining alleles. Our estimate of the minimum number of mates for this focal female was this resulting number (i.e. remaining alleles divided by two, rounded up, plus 1 (i.e. last male). Note that incorporating the last male genotype can improve this conservative estimate of the minimum number of mates compared to pure allele counting when the last male is homozygous: an array of four alleles including a homozygous last male, gives a minimum estimate of three males, while pure allele counting and dividing by two would have produced a minimum estimate of only two males. We so obtained four estimates (from

the four microsatellite loci amplified) of the minimum number of males present in a spermatheca, and used the greatest number of them as our estimate of the minimum number of males present in a spermatheca.

Our second method applied the probabilistic technique described in Bretman & Tregenza (2005) to estimate the number of males within each spermatheca. This technique uses population allele frequencies to determine the probability of observing a certain array of alleles if a different number of males contribute to this array. The probability of not observing an allele is $P_{\text{not observed}} = [1\text{-}f(a)]^t$, where f(a) is the allele frequency and t is the number of attempts at observing the allele, which is twice the number of males. The probability of observing an allele is $P_{\text{observed}} = 1 - P_{\text{not observed}}$. The probability of obtaining the observed array of alleles is calculated as the product of P_{observed} for alleles present in the array and $P_{\text{not observed}}$ for those alleles in the population that are not present in the observed array. We implemented this formula in a short program in MATLAB version 7.8 and calculated the probability of receiving the observed array of alleles if a female mated with 1 to 50 males, representing t = 2 to 100 (Bretman & Tregenza 2005). We did this for all four loci for all spermathecae. The number of attempts with the highest probability indicates the most likely number of males that generate the observed array. For our mixed model analyses (see below), we used the estimate derived from the most polymorphic locus (not necessarily the same locus for all spermathecae).
In addition, we checked the sperm from the last male for the occurrence of intraejaculate sperm sorting, i.e. if the different sperm from a heterozygous last male was found in the same spermatheca or in different spermathecae.

Statistical Analyses

We analysed the influence of season and other variables on the binary response variable female multiply mated (yes or no, i.e. if females had sperm from a single male or from several males within their sperm stores) with generalized linear models in R version 2.6.2 using the *glm* function from the *stats* package (R Development Core Team, 2008). We preferred analyses with a binary response variable over analyses with proportion data, because we had unique values of different explanatory variables for every individual case (Crawley 2007). Generalized linear models were fitted with binomial errors and logit link function. The explanatory variables included day in the spring season when flies were caught, female size, last male size, tears and notches combined, and large wing injuries. We started model simplification with a maximal model that included higher powers of the

explanatory variables and all two-way interactions. We performed model simplification based on information-theoretic approaches (Akaike Information Criterion AIC) and by using deletion tests (Chi-squared tests).

We analysed the influence of season and other variables on sperm storage (e.g. number of males detected within sperm stores) with linear mixed models in R version 2.6.2 (R Development Core Team, 2008) using the lme function from the nlme package (Pinheiro *et al.* 2008). Linear models are preferred over generalized linear models when the variance increases with the mean on the original scale of measurement (Crawley 2007). We therefore log10 transformed the response variable instead of using generalized linear mixed models with Poisson errors and log link. The explanatory variables included the day in the spring season when females were caught, the spermathecal identity (i.e., was the focal spermatheca the singlet, middle doublet, or outer doublet), female size, last male size, tears and notches combined (as an index of female age), and large wing injuries. Since non-parametric smoothers in generalized additive models clearly indicated curvature in the relationship between number of males detected in the sperm stores and day in the year when flies were caught (data not shown), we also included higher powers of day in the year (day^2 and day^3) as explanatory variables in our models. The random effects were the spermatheca nested within female. We performed model selection based on information-theoretic approaches (AIC) and by using hypothesis tests (i.e. testing simpler nested models against more complex models: likelihood ratio tests). Model selection and final model fitting was performed for both estimates (minimum estimate and the probabilistic estimate) of the response variable. Generalized linear models and linear mixed models were fitted by maximum likelihood (ML) during the process of model selection, while the final model was fitted by restricted maximum likelihood (REML).

Results

From the total 92 females collected in spring 2006, four females were not included in the final analyses because one female escaped from the vial before being frozen, one female had no sperm in her spermathecae, and two females had four spermathecae (and would have complicated our analysis of spermathecal identity). The occurrence of only two females with four spermathecae in our sample (2/92 = 2.2%) is considerably lower than previously reported for the same population (ca. 10%, see (Ward 2000).

We examined the remaining 88 females (3 x 88 = 264 spermathecae) to determine if they were

storing the sperm of the last male (that we had also collected). Although all males had copulated for ≥ 15 minutes with the female, in four cases we were not able to detect the alleles from the last male in any of the three sperm storage organs of the female. This finding could indicate that successful copulation does not always imply successful sperm transfer in the field (see Discussion). In the remaining 84 cases where we could detect the sperm from the last male in the female, the sperm was almost always present in all spermathecae (81 females). In three females the sperm from the last male was absent in one spermatheca: in one female in the singlet spermatheca and in two females in the outer doublet spermatheca. In addition, we screened all spermathecae of the females for the occurrence of intraejaculate sperm sorting of the last male (i.e., if the two different alleles of a heterozygous male are sorted and stored in different spermathecae). In all females we found no indication that females are able to distinguish and separate the sperm from one particular male: the two types of sperm produced by a heterozygous male were always found within the same spermatheca, or were always both absent from a particular spermatheca. Therefore, our study did not support intraejaculate sperm sorting and storage.

In 72 females (81.8% of females) we detected sperm from two or more males within the sperm stores. The remaining 16 females had sperm from only one male stored in their spermathecae: in 14 females this sperm belonged to the last male, and in two cases this stored sperm was from another male (i.e. not from the male that was captured together with the female). On average females stored sperm from 2.47 ± 0.13 (mean ± SE) males based on the minimum estimate, or 3.33 ± 0.24 based on the probabilistic estimate, respectively.

From the 264 genotyped spermathecae (dissected from 88 females), 10 spermathecae provided ambiguous arrays of alleles (i.e., very weak peaks). Since the replicated PCR runs of these spermathecae resulted in the same weak unreadable electropherograms, these 10 spermathecae obtained a NA (i.e., not available) in our mixed model analyses in R. Note that a missing value for a certain spermatheca did not imply an unavailable estimate for the other two spermathecae or the female as a whole, hence our sample sizes can differ for different results.

We analysed the incidence of multiply mated females (i.e. females that stored sperm from two or more males within their sperm stores) with generalized linear models with binary response variable, binomial errors, and logit link. The summary of the best model in terms of the Akaike Information Criterion (AIC) is given in Table 1. Only the quadratic and cubic term of day significantly influenced the incidence of multiply mated females (Table 1). The proportion of multiply mated

females sharply increased at the beginning of the spring season, stayed on a high level (> 90%) until mid June, and then decreased to ca. 60 % at the last sampling day (Fig. 1). Female size, last male size, tears and notches, large wing injuries, nor any interaction did explain if a females was multiply mated or not (Table 1).

We analysed sperm storage patterns (i.e., log10 transformed number of males detected within the sperm stores of females) with linear mixed models in R. Our procedure of model selection included hypothesis testing and information theoretic criteria. The summary of the best model in terms of the Akaike Information Criterion (AIC) for the minimum and probabilistic estimate of number of males detected is given in Table 2. The process of model simplification to obtain these models with the smallest AIC value included stepwise removal of the least significant interaction term. The models presented in Table 2 were achieved by removing interactions with $p > 0.30$, and none of the comparisons of models with progressively simplified fixed effects yielded a significant contribution of a single interaction term (all likelihood ratio tests: $p > 0.18$). Removal of the interaction spermatheca X female size from the model summarized in Table 2 caused a slight increase in the AIC value. Results from analyses with the probabilistic estimate of number of males detected within the spermathecae of females (Bretman & Tregenza 2005) were qualitatively not different from the analyses with the minimum estimate, with one small exception: the term spermatheca in the final model was marginally non-significant (Table 2).

Day of the year, large wing injuries, and spermatheca (in the more conservative model) significantly influenced observed sperm storage patterns (Table 2). Additionally, analyses revealed a significant interaction between spermathecal identity and the size of the last male in both models (Table 2). The three significant effects of day in the year (day in the year, quadratic term, and cubic term) highlight that sperm storage patterns and hence probably also sperm competition intensity strongly varies within the spring season (Table 2, Fig. 2). The number of males detected within the sperm stores of females continuously increases from April until the middle of June, before it abruptly decreases in the last one or two weeks dung flies are present at our sampling site in spring (Fig. 2). Sperm storage inferred from including population allele frequencies (i.e., the probabilistic estimate) unsurprisingly produced higher estimates of the number of males within the sperm stores of females, but showed the same seasonal pattern as the estimates obtained by the minimum approach (Fig. 2). The significant effect of spermatheca from the minimum estimate model (and the marginally non-significant effect in the probabilistic model) indicates a consistently lower number of sperm from different males for the singlet spermatheca (s1) compared to the middle doublet (s2)

and outer doublet spermathecae (s3) (Table 2, Fig. 3). Again, patterns inferred from both estimates were practically identical, although inclusion of population allele frequencies causes the difference between the middle doublet and outer doublet spermathecae to disappear (Fig. 3). In addition, the number of sperm from different males found within the spermathecae significantly increased with increasing number of large wing injuries a female has (Table 2, Fig. 4). This positive relationship between the number of males detected within the spermathecae of females and females' wing injuries suggests a mechanisms by which males may be able to assess females' mating history and/or prevalent sperm competition situation. The significant interaction between spermatheca and the size of the last male indicates an effect of the size of the last male on the sperm from different males detectable within the spermathecae: the bigger the last male the fewer males are detected within each spermatheca, but this decrease occurs differently in the three spermathecae (indicated by the different slopes of the lines, Fig. 5). The size of the female and tears and notches in wings (i.e., small wing injuries) did not explain sperm storage patterns.

Different types of wing injuries, tears and notches and the large wing injuries, changed considerably in abundance throughout the spring season (Fig. 6). Small injuries like tears and notches were more numerous than large wing injuries, and showed a peak in the middle (day 140) and at the end (day 173) of the sampling period (Fig. 6a). In contrast, large wing injuries exhibited only one peak in the middle of June (day 165: Fig. 6b).

Table 1 Summary of the generalized linear model for the binary response variable 'female multiply mated' (yes or no) as a function of day of the year flies were collected (including higher powers of day: day^2 and day^3), female size, last male size, tears and notches, and large wing injuries using restricted maximum likelihood (REML). Model was fitted with a binomial error structure and logit link function.

	df	Deviance	Residual df	Residual deviance	p value
Null			85	82.636	
Day of the year	1	1.510	84	81.126	0.21915
Female size	1	0.285	83	80.841	0.59335
Last male size	1	0.193	82	80.648	0.66083
Tears and notches	1	1.247	81	79.401	0.26414
Large wing injuries	1	1.222	80	78.179	0.26902
(Day of the year)^2	1	7.938	79	70.241	**0.00484***
(Day of the year)^3	1	4.793	78	65.449	**0.02858***
Day of the year X Large wing injuries	1	3.705	77	61.744	0.05425

*** Chi-square tests ignificant at the ≤ 5% level.**

Table 2 Summary of the linear mixed model for log10 transformed number of males detected in storage as a function of day of the year flies were collected (including higher powers of day: day^2 and day^3), the spermatheca, female size, last male size, tears and notches, and large wing injuries using restricted maximum likelihood (REML). The random effects part of the model comprised spermatheca nested within female.

			ME[1] as response		PE[2] as response	
Source	Numerator df	Denominator df	F value	p value	F value	p value
Intercept	1	151	344.6602	**< 0.0001***	295.7211	**< 0.0001***
Day of the year	1	77	14.7012	**0.0003***	13.3769	**0.0005***
Spermatheca	2	151	3.2412	**0.0419***	2.6795	0.0719
Female size	1	77	0.4950	0.4838	0.8452	0.3608
Last male size	1	77	1.9736	0.1641	2.5835	0.1121
Tears and notches	1	77	0.0978	0.7553	0.0961	0.7574
Large wing injuries	1	77	12.5939	**0.0007***	13.4472	**0.0004***
(Day of the year)^2	1	77	10.0765	**0.0022***	11.9623	**0.0009***
(Day of the year)^3	1	77	11.9123	**0.0009***	8.7569	**0.0041***
Spermatheca X Female size	2	151	0.9893	0.3742	1.2696	0.2839
Spermatheca X Last male size	2	151	3.3522	**0.0376***	4.3933	**0.0140***
Spermatheca X Tears and notches	2	151	2.7401	0.0678	1.5110	0.2240
Last male size X Large wing injuries	1	77	1.9745	0.1640	2.1774	0.1441

*** F-tests significant at the ≤ 5% level.**

[1] ME, minimum estimate.

[2] PE, probabilistic estimate.

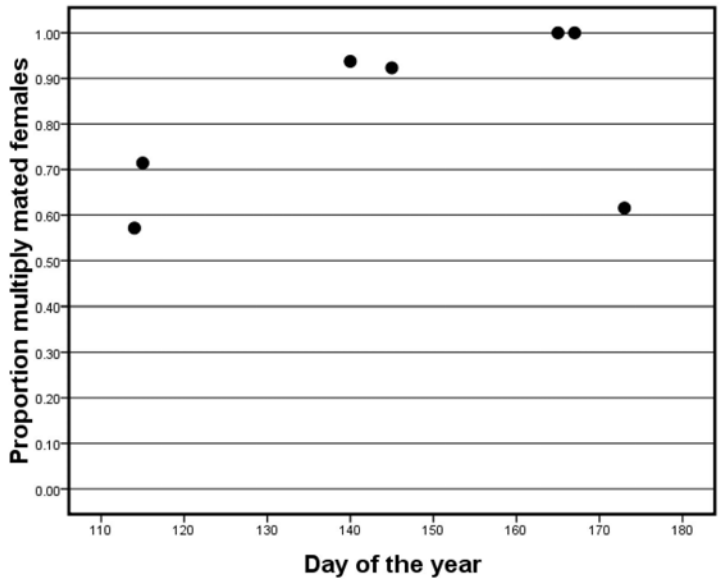

Fig. 1 Proportion multiply mated females (i.e. females with sperm from two or more males within their sperm stores) against day of the year. Sampling days from April to June: 24 April, day 114; 25April, day 115; 20 May, day 140; 25 May, day 145; 14 June, day 165; 16 June, day 167; 22 June, day 173.

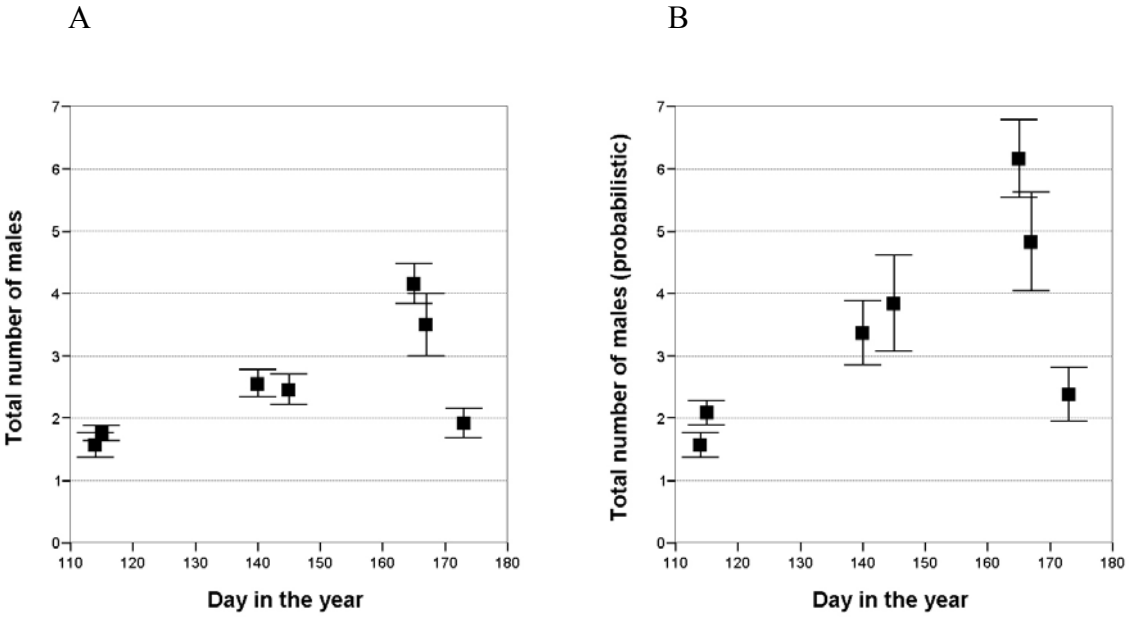

Fig. 2 A) Mean (± 1 SE) total number of males detected within the sperm stores of females over the spring season using the minimum estimate (see text for explanation). B) Mean (± 1 SE) total number of males detected within the sperm stores of females over the spring season using the probabilistic estimate. Sampling days from April to June: 24 April, day 114; 25April, day 115; 20 May, day 140; 25 May, day 145; 14 June, day 165; 16 June, day 167; 22 June, day 173.

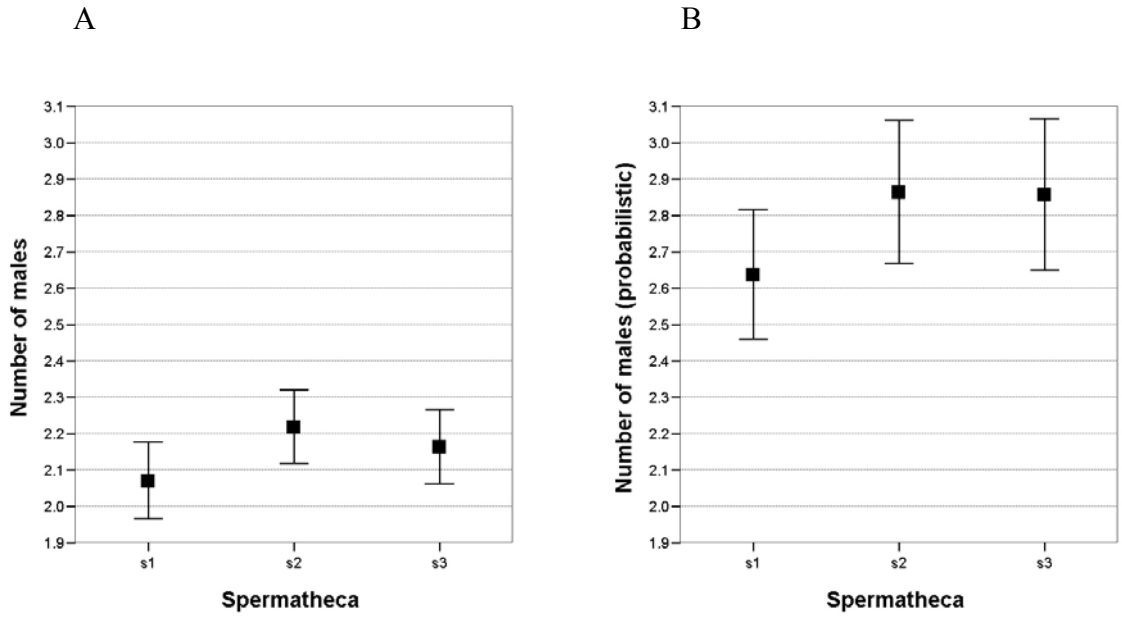

Fig. 3 A) Mean (± 1 SE) number of males detected within each of the three spermathecae using the minimum estimate (see text for explanation). B) Mean (± 1 SE) number of males detected within each of the three spermathecae using the probabilistic estimate. s1, singlet spermatheca; s2, middle doublet spermatheca; s3, outer doublet spermatheca.

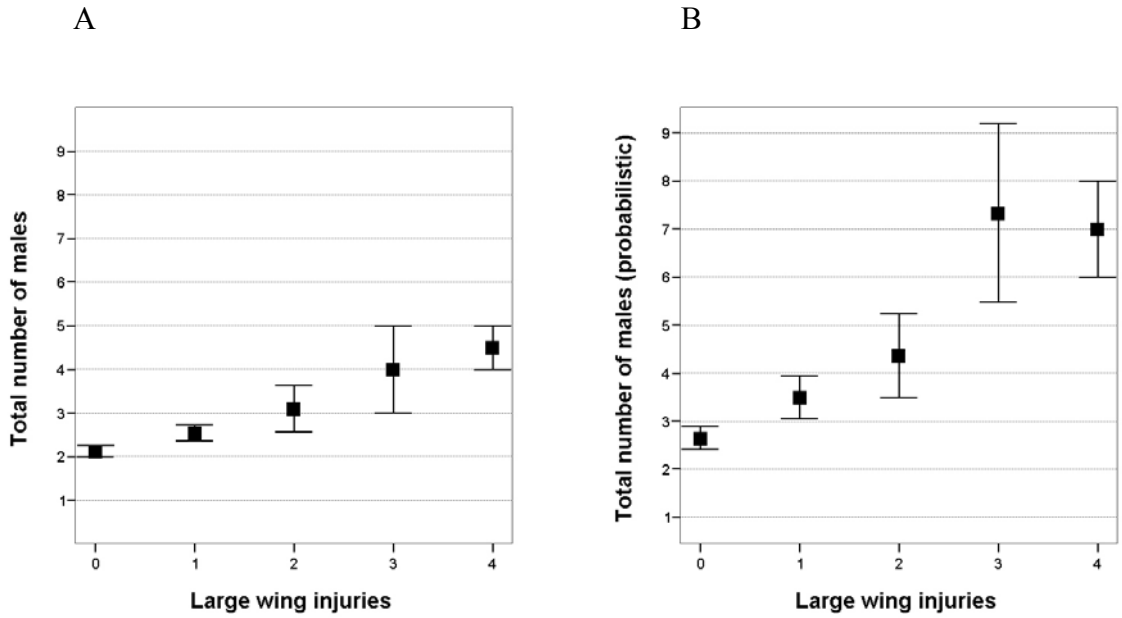

Fig. 4 A) Mean (± 1 SE) total number of males detected within the sperm stores of females against large wing injuries using the minimum estimate (see text for explanation). B) Mean (± 1 SE) total number of males detected within sperm stores of females against large wing injuries using the probabilistic estimate.

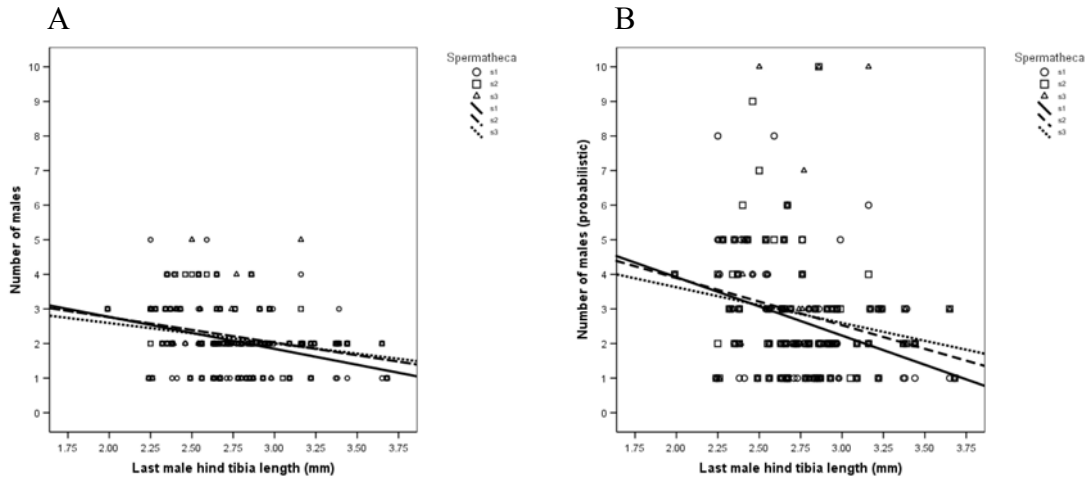

Fig. 5 A) Number of males detected within each spermatheca against the size of the last male using the minimum estimate (see text for explanation). B) Number of males detected within each spermatheca against the size of the last male using the probabilistic estimate.

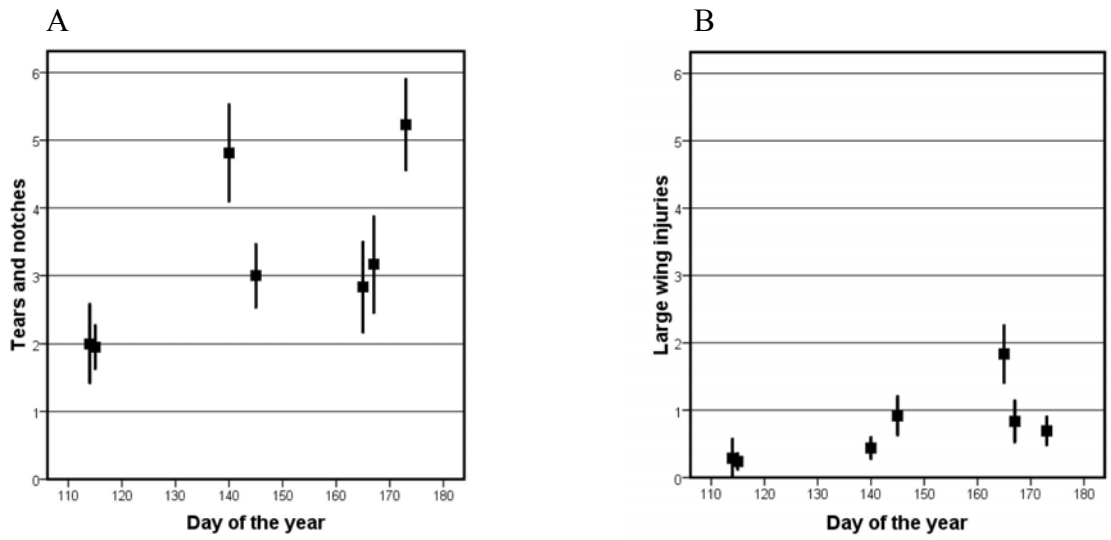

Fig. 6 A) Mean (± 1 SE) number of tears and notches and B) large wing injuries over the spring season (see text for explanation).

Discussion

Our study provides estimates of levels of polyandry, as well as temporal changes in sperm competition intensity for a natural population of yellow dung flies *S. stercoraria*. Polyandry has tremendous evolutionary consequences and field data such as ours are essential to accurately test sexual selection and sexual conflict theory under laboratory settings. Unfortunately, field data in this context are still scarce. To our knowledge, only one other study documented temporal changes in sperm competition intensity in a natural population of non-social insects (Simmons et al. 2007). Interestingly, by highlighting that large injuries on the wing of females explain the number of different ejaculates that are already stored within the spermathecae, our study provides an optical cue by which males could potentially easily assess sperm competition intensity before or during matings. Furthermore, our data demonstrate that the number of ejaculates in storage differ amongst spermathecae in wild yellow dung flies. Such differential sperm storage could represent the basis by which females can influence paternity at the time of fertilization, by differentially utilizing sperm from the three spermathecae (Hellriegel & Ward 1998).

Last male sperm and intraejaculate sperm sorting

In four of the 88 females we could not detect the sperm of the last male in the sperm stores, although all females had copulated for at least 15 minutes with these males. This finding could indicate that in nature not all copulations lead to successful sperm transfer. A recent review showed that rates of non-sperm representation due to insemination failures or other reasons may be high across insect species (Garcia-Gonzalez 2004). One possibility is that in certain females the sperm from the last male had not yet reached the spermathecae because of the abbreviated copulations (>15 min). Copulations normally last around 35 minutes, though copula duration decreases with repeated matings (Parker 1970d). P_2 (the proportion of paternity assigned to the second of two copulating males) increases with copula duration (Parker 1970d). Similarly, S_2 (the proportion of stored sperm assigned to the second of two copulating males) in the spermathecae increases with copula duration (Simmons et al. 1999). However, mean S_2 in the spermathecae after 15 minutes is only about 35% and associated with considerable variation (see Figure 3B in (Simmons et al. 1999)), emphasising the possibility that in certain copulations no sperm of the second male (respectively last male) is found in the spermathecae after ca. 15 minutes. A current study investigating sperm storage patterns in singly-mated females where copulations have been

interrupted after 20 minutes confirms this: in some spermathecae no sperm of the male is present (C. Wüst, M. Demont, C. Buser, and L.F. Bussière, unpublished data). Unfortunately, in the present study we are unable to distinguish between failed inseminations, successful inseminations in which the sperm did not yet reach the spermathecae, or cryptic female choice against certain sperm.

Since virtually all captured males were heterozygous at one or more loci, we were able to investigate whether intraejaculate sperm sorting occurs in yellow dung fly females (i.e. if different alleles from the same male are stored in different spermathecae). Intraejaculate sperm sorting could facilitate intraejaculate sperm selection, which can be beneficial in certain situations, for example by producing offspring of one particular sex (Simmons 2001). Yellow dung fly males show greater mortality at high temperatures than females (Ward & Simmons 1990), potentially causing changes in natural sex ratios, and providing a specific situation in which it may pay females bias offspring sex ratios towards males. Stockley and Simmons (1998) showed that females that used previously stored sperm to fertilize their eggs produced significantly higher ratios of male to female offspring than females which displaced their sperm before oviposition. Ageing of sperm in the female sperm storage organs or adaptive intraejaculate sperm selection could be responsible for this pattern (Stockley & Simmons 1998). In the present study, we found absolutely no evidence for intraejaculate sperm sorting in yellow dung flies: both alleles of a heterozygous male were always either present in or absent from a particular spermatheca. As the applied microsatellite loci are not located on either sex chromosome (Demont et al. 2008; Garner et al. 2000), this conclusion only refers to autosomes in yellow dung flies. The possibility of intraejaculate sperm sorting and selection based on sex chromosomes remains to be evaluated in this species.

Level of polyandry and temporal changes in sperm competition intensity

Our study revealed high levels of polyandry in a natural population of yellow dung flies: 81.8% of females stored sperm from two or more males within their sperm stores. On average 2.47 or 3.33 ejaculates compete within the sperm storage organs of wild flies, based on the minimum or probabilistic estimate, respectively. Studies investigating direct or indirect benefits of polyandry in this species (Hosken et al. 2003; Tregenza et al. 2003) and studies investigating evolutionary responses to polyandry (Hosken 2001; Hosken et al. 2001; Hosken & Ward 2001) used laboratory settings in which females were mated to two or three males. The results from the present paper suggest that investigating the causes and consequences of polyandry in dung flies with double matings and threefold matings is a reasonable starting point, since on average two to four ejaculates are found at the same time within the spermathecae of females. However, our study also clearly

revealed substantial variation in the number of ejaculates stored within wild females, and that the number of ejaculates stored within females (i.e., sperm competition intensity) exhibits strong temporal variability. The number of ejaculates detected within females increased continuously over the spring season reaching a peak in the middle of June, and then declined rapidly and drastically. The most likely explanation for this pattern is that females mate repeatedly with different males and continuously accumulate sperm from different males as they get older (but see below). The decline in the second half of June could arise because only young flies from the second spring generation (who have mated fewer times) are still present on cow pats. Surprisingly, our study detected an increase in the number of stored ejaculates until mid June (14th June: highest number of males detected within spermathecae), but the second spring generation adults are assumed to be on the pasture from the middle of May onwards (Blanckenhorn et al. 2001). Our data on minor damage to wings (tears and notches, *not* large injuries) would support this point in time, as the frequency of damage decreases sharply in the second half of May. If the number of sperm from different males stored by females is assumed to be primarily a function of female age, then the arrival of new young females of the second spring generation would actually cause a decrease in the number of ejaculates competing within females from the middle of May (i.e. earlier than we observed). This is because from mid-May samples would consist of both old females from the first spring generation and young females from the second generation. However, the sample from 14th June (day 165) only comprised females that had stored sperm from three or more males (i.e., no females that were just mated once or twice). The relatively small sample size on this date (n = 12) could explain the observed pattern if the ratio of old to young females would be biased towards old females, and we therefore only collected old females by chance. However, the occurrence of females with relatively few tears and notches on their wings on this day contradicts this scenario. The pattern of tears and notches indicates that old and young females are present at 14th June (day 165) and all of them have already mated many times.

At the end of the spring season (last sampling day), only young, or alternatively females that have not yet mated with many males, were found on and around cow pats. Assuming that tears and notches reflect regular wear (so are a useful indicator of age) and large wing injuries rather intra- or inter-specific interactions (so may indicate "activity") as proposed by Burkhard and colleagues (2002), then the last females present on cow pats at the end of the spring season are old flies that have not been very active (cf. Fig. 6). At present these are only speculations, but the possibility that sperm storage patterns (e.g. number of mates) are determined to a greater extent by activity (= frequency of large wing injuries) rather than directly by age (= tears and notches) is a fascinating

idea, yet one which requires further investigation.

These temporal changes in the number of ejaculates represented within females and the associated temporal changes in sperm competition intensity have three important implications. First, our finding of 2.47 or 3.33 different ejaculates on average within the sperm stores of females arises from genotyping both young and old females. Consequently, young females (or alternatively "less active" females, see above) tend to show a lower than this average level of sperm competition intensity, and older females a higher level. Our data also demonstrated that females mate with up to 6 or 11 males in the field based on our two different estimates. These estimates are in good agreement with an earlier study, which reported that females maximally produce seven clutches of eggs in the field (Gibbons 1987). This is a much higher level of polyandry than commonly applied in laboratory investigations in yellow dung flies. Future research should investigate costs and benefits of polyandrous behaviour when females are mated across the whole range of multiple matings observed in natural populations (e.g. two matings, four matings, six matings), perhaps complemented with experimental evolution applying different polyandry levels in yellow dung flies. Second, the increased sperm competition intensity as the spring season advances contrasts sharply with precopulatory sexual selection patterns in this species. Density measured as number of males per pat is highest at the beginning of spring and then decreased significantly over the spring season. The operational sex ratio measured as the number of females divided by the number of males at the mating site is likewise lowest at the beginning of spring and then increases significantly over the spring season, indicating that more females per male are available in late spring (Jann et al. 2000). Jann and colleagues (2000) additionally showed that precopulatory male-male competition increased with competitor density and consequently decreased over time in spring. In contrast, the present study showed that sperm competition intensity increased over time in spring before it declined abruptly at the end of the spring season. Therefore, yellow dung flies are confronted with a situation of high levels of precopulatory male-male competition and low levels of sperm competition early in spring, and exactly the opposite pattern late in spring. Postcopulatory sexual selection intensity for male paternity success remains to be established in yellow dung flies, and this might best be achieved by also investigating temporal patterns of variation. Temporal variation in the intensity and direction of precopulatory *and* postcopulatory sexual selection could contribute to the maintenance of genetic diversity in this species. Third, the pronounced temporal changes in the number of ejaculates present within the spermathecae of females could also greatly influence sperm investment by dung fly males. Several studies have reported evidence for males ejaculating strategically depending on the risk or intensity of sperm competition or depending on the female

"quality" or "condition" (Wedell et al. 2002). In some of these studies, scientists were also able to uncover how males detect female mating status or sperm competition intensity (Carazo et al. 2004; Thomas & Simmons 2009). Further it has been shown that this plastic responses to the level of sperm competition can result in increased male reproductive fitness (Bretman et al. 2009). In yellow dung flies, female size influences investment in ejaculate size: males copulate longer with larger females (Parker et al. 1999). In contrast, until now no study specifically investigated strategic sperm allocation in yellow dung flies according to sperm competition intensity or the presence of rival males. However, the present data clearly indicate that sperm competition intensities change strongly over the season. Furthermore, our study revealed that there is a link between the number of large injuries on the wings of females and the number of ejaculates the female had stored within their spermathecae, potentially providing the males with an obvious cue to assess sperm competition intensity. It would be interesting to manipulate female mating history and wing injuries of females independently of each other, and to investigate how males respond to them (e.g. in terms of copula duration or sperm transferred). In addition, it would be interesting to see if the seasonal changes in sperm competition intensity are reflected in seasonal changes in investment in reproductive tissue (e.g. testis) or copula duration.

Biases in sperm sorting

The process by which a female has a mixture of sperm from different males in her sperm storage organ(s) and selectively uses those sperm from a particular male at the time of fertilization is referred to as sperm selection (Simmons 2001). It is the most cryptic and most controversial mechanism of female choice, in part because few studies have documented evidence in favour of it. Ward (2000) found indications that yellow dung fly females are able to select sperm on the basis of their phosphoglucomutase PGM genotype. Fitting offspring PGM genotype to environmental conditions has the potential to increase offspring performance. However, not all predictions from an adaptive sperm selection scenario were supported in that experiment (Ward 2000), raising questions about the precise extent or context in which females can exert sperm selection in yellow dung flies. A recent population genetics study contributed to this controversy by showing that PGM is neutral (i.e. not under selection) in yellow dung flies (Demont et al. 2008). Nevertheless, differential sperm storage across the spermathecae as found here has been suggested to be a necessary prerequisite for a female to exert sperm selection (Hellriegel & Bernasconi 2000; Hellriegel & Ward 1998). If sperm from different males is stored in different proportions across the spermathecae, then females may subtly influence paternity by preferentially utilizing sperm from a certain spermatheca at the

time of fertilization. Laboratory experiments clearly indicate that sperm is stored in different proportions across the spermathecae and that the time elapsed between copulations might be critically important in determining sperm sorting (Bussiere et al. 2009). In agreement with these laboratory findings, the present study, as well as another field study (Chapter 5), showed that the number of ejaculates stored differs amongst spermathecae in wild flies as well. Taking into account sperm storage patterns from the last male (see above) and results from Bussière and colleagues (2009), it seems that the sperm from the last male is usually stored in different proportions across the spermathecae (sometimes resulting in a lack of sperm in one spermatheca) and that these sperm are subsequently displaced by further mates. Different proportions of sperm in the spermathecae that are differentially displaced by subsequent sperm results in the sperm storage pattern described in the present study: different number of ejaculates present in the spermathecae. Spermathecae consisting of unequal sperm mixtures could indeed enable females to bias paternity towards certain males. But further studies are needed to investigate when and to what degree this is possible (cf. Chapters 3, 5).

The significant interaction between spermatheca and the size of the last male indicated that the bigger the last male was the fewer males are detectable within the spermathecae and that this effect is differentially strong for the three spermathecae. The decrease in the number of ejaculates present within the spermathecae could be explained by the previously documented higher rates of sperm transfer of large males in this species (Parker & Simmons 1994; Parker & Simmons 2000). The subtle difference in slope could arise because spermathecae differ in size (Chapter 3).

Our study provides essential estimates of levels of polyandry and temporal changes in sperm competition intensity for a natural population of yellow dung flies. Our data also demonstrated a specific and simple optical cue (i.e. large wing injuries) through which males could assess prevailing sperm competition intensity, and additionally showed that the number of ejaculates in storage differ amongst spermathecae of wild female yellow dung flies. Field data as presented here could establish the basis for subsequent detailed studies on sperm storage and utilization, strategic ejaculation, cues indicating sperm competition intensity, and the comparison of pre- and postcopulatory sexual selection. More generally, such data can further help improve laboratory settings for investigating polyandry and associated aspects of sexual selection. Summarized, field data on multiple mating, sperm storage, postcopulatory processes and paternity is not just a welcome complement to laboratory data, but crucial in order to acquire a more complete understanding of sexual selection.

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Chapter 5

Wild yellow dung fly females benefit from polyandry but show no evidence of sperm selection based on dung pat microclimate

Marco Demont

Abstract

Over the past decade, new molecular techniques have substantially improved our knowledge of postcopulatory sexual selection (sperm competition and cryptic female choice). Nevertheless, studies that examine patterns of sperm utilization in natural populations of nonsocial insects are rare, support for sperm selection (active and adaptive biases in the use of stored sperm) is still elusive, and its relevance within natural populations unknown. I performed an oviposition site choice experiment in the field in which female yellow dung flies *Scathophaga stercoraria* (Diptera: *Scathophagidae*) could deposit their eggs into three different micro-environments on a dung pat (the east-west ridge, north- or south-exposed side), and genotyped the offspring and sperm remaining after oviposition from the three spermathecae of these flies. Temperature strongly influenced egg placement: the warmer the temperature, the higher the proportion of eggs laid into the north-exposed side of dung. The number of ejaculates in storage differed amongst spermathecae and females stored sperm from more males than fathered their offspring. On average, 2.11 sires (range: 1 – 3) were detected in the genotyped clutch, while females carried sperm from 2.84 males (1 – 5) within their sperm stores. Mean last male paternity was 83.4 %, roughly matching previous estimates from the laboratory. Importantly, I found no indication that females are able to lay eggs of different genotypes, by biasing paternity towards certain males, in different places. Thus, my study failed to demonstrate adaptive sperm selection. However, my study revealed positive effects of multiple mating on the total number and proportion of offspring emerging. I discuss these findings in the context of postcopulatory sexual selection and argue that an integration of field studies similar to my work and controlled laboratory experiments are essential to promote our understanding of polyandry and cryptic female choice.

Introduction

The fitness benefit of multiple mating is much more obvious for males than for females: more mates confer higher fecundity in males, but not necessary in females. In addition, multiple mating is often remarkably costly for females (Arnqvist & Rowe 2005). Nevertheless, females of most organisms are polyandrous (mate with more than one male), a pattern for which we still lack a comprehensive explanation. Among the contending explanatory factors, direct and indirect (genetic) fitness benefits have been suggested to account for the evolution and maintenance of polyandry, but despite a substantial amount of theoretical and empirical work in this context, our understanding of the causes and consequences of polyandry is still fragmentary (Simmons 2005; Tregenza & Wedell 2000). The fact that research on polyandry is primarily focused on laboratory experiments, even though we often do not know how well this reflects natural conditions, impedes progress in this field of research. More studies of polyandry in wild populations are clearly needed (Bretman & Tregenza 2005).

Measuring the degree of polyandry by tracking females and directly observing matings can be difficult for insects. But sperm storage in female insects is almost ubiquitous, and genotyping these sperm stores can successfully provide useful information on female mating frequency in natural populations. Investigating how sperm storage translates into paternity and how many fathers contribute to a clutch of offspring is one farther issue that needs to be addressed in the study of polyandry and postcopulatory sexual selection. Most of the previous work in this area has been conducted on social insects, but recently a few studies have broken new ground in extending this work to other taxa (Bretman & Tregenza 2005; Simmons et al. 2007). These studies are particularly important for evaluating if sperm utilization patterns demonstrated with double matings in the laboratory are sustained following multiple matings. The existing data provide equivocal validation of these lab methods: some studies show the same paternity patterns (e.g. last male paternity) for double and multiple matings (Cobbs 1977; Simmons 2001), while in other cases sperm were used differently after multiple matings as compared to twice mated females (Lamunyon 1994; Simmons et al. 2007; Zeh & Zeh 1994). More work integrating these studies or extending them to new systems is clearly needed (Simmons 2001).

Polyandry can give rise to postcopulatory sexual selection, and numerous mechanisms of sperm competition and cryptic female choice have been described (Birkhead & Pizzari 2002; Eberhard 1996; Simmons 2001; Snook 2005). While sperm competition is generally seen as a strong selective agent (Parker 1970), the extent and importance of certain mechanisms of cryptic choice by females are the subject of considerable debate. The most cryptic mechanism of postcopulatory female choice is sperm selection (Simmons 2001). Sperm selection is the process of selective utilization of certain sperm by females at the time of fertilization, when they have a mixture of sperm from different males in their sperm store(s) (Simmons 2001; Simmons & Siva-Jothy 1998). Convincing evidence for sperm selection is extremely scarce. One requirement is doubtless a precise understanding of all (other) mechanisms enabling and enacting cryptic female choice: sperm reception, transport within the female reproductive tract, and storage. Additionally, a convincing demonstration of adaptive sperm selection would also require an estimation of the indirect benefits (e.g. good genes or compatible genes).

One of the most compelling systems for which there is some evidence of sperm selection is the yellow dung fly, *Scathophaga stercoraria* (Ward 2000). The yellow dung fly is a naturally polyandrous species. Experiments with single and double mated yellow dung fly females revealed no simple benefits or costs of multiple mating (Tregenza et al. 2003), but a study in which females mated once or three times revealed a longevity cost to females that copulated with more males (Hosken et al. 2002). Potentially offsetting this cost of mating, there is also some evidence that indirect benefits can be acquired by polyandrous mating: males that were more successful in sperm competition also had offspring that developed faster (Hosken et al. 2003). However, experimentally enforced polyandry and monogamy have rapid and strong evolutionary consequences in *S. stercoraria*. Polyandrous lines invest more in reproductive tissue, testes and female reproductive accessory glands (Hosken et al. 2001; Hosken & Ward 2001), but have decreased immune function (Hosken 2001). Furthermore, results from a study investigating the fitness consequences of females evolving under enforced monogamy or polyandry when mating once, suggested that sexual conflict rather than a pure good-genes scenario may drive evolution under enforced polyandry (Martin et al. 2004).

Yellow dung fly males aggregate on and around dung pats where copulations take place. There is strong male-male competition, and several studies have found strong mating advantages for large males (Jann et al. 2000). During subsequent oviposition on cow pats, the males guard their female mates. Females prefer to lay their eggs on small hills on the dung surface and avoid depressions and

sharply elevated points that imply a higher risk of drowning or drying-out, respectively, and such female choice of suitable oviposition sites increases female reproductive success (Ward et al. 1999). Findings regarding egg density are inconsistent: Ward *et al.* (1999) found that oviposition was not influenced by the presence of other eggs, a recent study in contrast, found that females do respond to egg density (especially large females) by decreasing clutch size on crowded pats (Claudia Buser, unpublished data). Intriguingly, females seem not only to choose where to lay their eggs, but also what kind of eggs they lay. In a series of studies Ward and coworkers suggested that females are able to match the phosphoglucomutase (PGM) genotypes of their offspring to the prevailing environmental conditions. Females collected in the field and allowed to oviposit in the laboratory produced offspring of different PGM genotypes depending on environmental conditions: one PGM allele was relatively more common if eggs had been laid in simulated sunshine (light bulb), and another PGM allele was relatively more common if the eggs had been laid in simulated shade (no light bulb) (Ward 1998). In the same study, Ward (1998) showed that heterozygotes at the PGM locus grew better (i.e. showed higher pupal weight) in a variable temperature treatment, while homozygotes grew better at constant temperature. These data on pupal performance suggest that females could potentially increase the fitness of their offspring by biasing paternity towards males with certain PGM genotypes, depending on which environment females lay their eggs. Predictions were partly confirmed by a study in which females homozygous for the most common PGM allele were mated with two homozygous males of the same or different genotype as the female. Again, males with the same genotype were indeed more successful in gaining paternity with females that experienced the constant temperature, but homozygous males with a different genotype (hence producing heterozygous offspring) achieved no higher paternity than males with the same genotype (hence producing homozygous offspring) in the variable environment (Ward 2000). Nevertheless, these experiments suggested that sperm selection might occur in yellow dung flies, but to date it appears that the phenomenon is restricted to a fraction of females and environmental circumstances (Ward 1998; Ward 2000). One study tried to link these laboratory findings to natural populations, finding that PGM alleles from egg samples were non-randomly distributed between north and south slopes and between shaded and sunny parts of artificial cow pats in the field (Ward et al. 2002). However, Ward et al. (2002) could not distinguish whether the same females laid eggs of different genotypes in different places by selectively choosing their paternity, or whether females of different genotypes laid their eggs in different places.

For practical reasons studies on sperm competition and sperm utilization often suffer from one of the following three limitations: 1) Mates are randomly assigned, which eliminates precopulatory

sexual selection; 2) outcomes of copulation and fertilization are observed in isolation (e.g. no other animals present); 3) mating pairs are disturbed during copulation or oviposition (e.g. transferring mating pairs during copulation on a substrate where oviposition can occur). In the present study, I tried to minimize these potential influences on the outcome of postcopulatory sexual selection in order to study sperm storage and paternity patterns in as natural a situation as possible. In particular, I addressed the following questions: i) Does the number of ejaculates in storage differ amongst spermathecae? ii) Do wild yellow dung fly females mate with more males than sire their offspring? iii) Does last male paternity in the field accord with previous estimates from the laboratory? iv) Do females exert sperm selection based on dung pat microclimate? v) Do females benefit from polyandrous behaviour (i.e. number of ejaculates detected within their sperm stores) in terms of fecundity or fertility?

Materials and Methods

Field work

I sampled a total of 22 dung fly females on four days in May 2006 on a pasture in Fehraltorf, near Zurich, Switzerland (8.55°E, 47.37°N). I collected fresh cow dung on the pasture, formed small artificial dung pats on Petri dishes (diameter: 9 cm), and distributed these dishes throughout the pasture. Because I wanted to vary the microclimate of these artificial pats to mimic the natural situation, all dung pats had a “roof shape” with a raised east/west ridge in the middle. Consequently, females could lay their eggs into three distinct areas (micro-environments) on the pat: the ridge, the south exposed surface, or the north exposed surface. I carefully covered the Petri dishes with a cage (dimensions: 29 x 29 x 29 cm) as soon as a female started oviposition on the artificial dung pat. Thus I did not assign males and females to each other, I did not disturb copulations, and other yellow dung fly males were present during copulation and oviposition. Dung pat age was defined as the time between distributing the pats on the pasture and the time when oviposition started. I measured temperature in the sun (not shade) close to the dung pat during oviposition. After oviposition, I passed a collecting vial through a sleeve in the cage and collected the focal female(s) (in two cases, two females were present on the pat at the same time) and one or more associated males. I recorded the dung pat from which each fly was captured. Flies and dung pats were subsequently brought to the laboratory.

Upon arrival in the laboratory, adult flies were immediately frozen at -80°C, and I counted the number of eggs laid in the north (N), south (S) and ridge (R) areas. I then transferred eggs according to their microclimate-origin into 200 ml plastic rearing containers (one container per clutch and origin). Transferred eggs were raised in climate chambers at constant 20°C, 60% relative humidity, and 13 h light: 11 h dark regime. I checked the containers for emerged adults every day until no individuals emerged for three weeks. All emerged flies were immediately frozen at -80°C and subsequently genotyped.

Dissections

I extracted sperm from the spermathecae using a method described by Tripet and colleagues (2001) and applied before in yellow dung flies (Bussiere et al. 2009). I separated the abdomens of the dung fly females from the rest of the body and stored them for 48 hours in 70% ethanol. Under a quality binocular microscope (Leica MZ-12, Leica Microsystems GmbH, Wetzlar, Germany) I afterwards carefully removed the posterior part of the female reproductive tract (including the common oviduct, spermathecae, spermathecal ducts, accessory glands, and the bursa copulatrix) by grasping the genital valves in forceps and tearing them from the abdomen (cf. Bussière *et al.* 2009). Next, I separated the three spermathecae individually from the rest of the reproductive system and transferred them individually to a drop of distilled water. For every female, I could easily distinguish the singlet spermatheca (regardless of the side of the body on which it is found) from the middle and outer doublet spermathecae (Hosken et al. 1999). I removed all tissue that surrounded the spermatheca and then applied soft pressure to the spermathecal capsule. In this way, I carefully broke the spermatheca open, and since the storage in 70% ethanol caused the ejaculate in the spermatheca to coagulate I could remove the sperm pellet from every single spermatheca (Tripet et al. 2001). The three sperm pellets from each female, each originating from a different spermatheca, were transferred to 180 µl of buffer solution (ATL buffer from the QIAamp® DNA Micro Kit, Qiagen; see below) and immediately stored at -80°C for subsequent DNA extraction.
I photographed and measured the hind tibiae of all flies under a binocular microscope with the software ImageJ 1.37v (Wayne Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/).

Extraction, amplification and analysis of DNA

I performed DNA extraction from sperm pellets according to Bussière *et al.* (2009): I used a kit

designed for small amounts of DNA sample (QIAamp® DNA Micro Kit, Qiagen AG, Switzerland) to extract the potentially very low number of DNA copies from sperm pellets, and I added carrier RNA to buffer AL (1 µl dissolved carrier RNA in 200 µl buffer AL). Note that carrier RNA does not dissolve in buffer AL; it must first be dissolved in buffer AE and then added to buffer AL. I used the minimum recommended amount of elution buffer AE (20 µl) to retain the highest possible concentration of sperm DNA. I used the QIAGEN® Multiplex PCR Kit (Qiagen AG, Switzerland) to simultaneously amplify seven microsatellite loci: SsCa1, SsCa3, SsCa16, SsCa21, SsCa24, SsCa26, and SsCa30 (Demont et al. 2008; Garner et al. 2000). Total PCR reaction volume for the sperm was 30 µl (cf. Bussière *et al.* 2009 used only 24 µl): 5 µl DNA template, 15 µl QIAGEN Multiplex PCR Master Mix, 7 µl distilled water and 3 µl microsatellite primer mix (100 µM). Cycling conditions for the sperm were as follows: 95°C for 15 min, then 30 cycles of 94°C for 30 s, 60°C for 3 min and 72°C for 45 s, and finally 60°C for 30 min. These cycling conditions did not produce large stutter peaks for six of the applied markers. Locus SsCa21 was the exception, consistently showing stutter. This was not a problem for paternity analyses since I could match offspring genotypes to parental genotypes. In contrast, stutter peaks could potentially cause problems for quantifying sperm storage patterns (i.e. number of males detected within spermathecae). I therefore excluded SsCa21 from sperm storage analyses.

I used a Chelex extraction method to extract DNA from the heads of all flies (parents, offspring, and other flies that were collected from the artificial cow pat). Heads were transferred into 96-well PCR plates kept on ice. I then pipetted 100 µl of 6 % Chelex suspension (Chelex 100®, Na^+-form, particle size 50 – 100 mesh, Fluka) into each well using wide-ended tips. Afterwards I covered the plate with a plastic mat, carefully shook it, and spun down the heads to ensure sample in liquid. I used a thermocycler to incubate plates 60 minutes at 55°C, boil 9 minutes at 100°C, and cool down to 20°C. After taking samples out of the thermocycler I again shook them carefully and spun them down, before the plate was stored at 4°C for 10 to 20 hours, and afterwards frozen at minus 20°C for at least 24 hours before DNA extractions were used for subsequent processing. DNA template amount (1µl), total PCR reaction volume (6µl), and cycling parameters (number of cycles: 27) for the heads were the same as in Bussière *et al.* 2009.

All PCR products from sperm and heads were separated on a capillary sequencer (Applied Biosystems 3730 DNA Analyzer), and the output analysed using Applied Biosystems GeneMapper® software. Genotypes from heads were simple to score. Sperm samples were more challenging because of the number of alleles present. To avoid artificial inflation of my estimate of

the number of alleles and males present in the sperm stores, I did not consider small peaks on either side of a large peak since they could potentially represent stutter peaks. The only exceptions were small peaks (alleles) that were also found in the offspring: those were counted. I obtained the number of alleles present in every spermatheca by counting the alleles after I had discounted all alleles that could potentially come from the female (in case of incomplete removal of female tissue during dissection). I obtained the number of males present in every spermatheca by applying the following procedure. In cases when maternal alleles were present in the array of alleles, these were discounted. I then identified the alleles from the last male in the array and subtracted them. I afterwards divided the remaining alleles by two (rounding up) because every male could potentially be heterozygous. My estimate of the minimum number of males was then this resulting number plus 1 (i.e. the last male). I therefore obtained separate estimates of the minimum number of males present in a spermatheca from the six microsatellite loci amplified (i.e. locus SsCa21 excluded), taking the largest number as my estimate of the minimum number of males present in any given spermatheca.

P_{last} (the proportion of paternity assigned to the last male mated to a female) was estimated as follows: I determined by subtraction which alleles were passed on by the male. If an offspring had the same genotype as the mother, then the exact paternal contribution for that locus is unclear (e.g. one or the other allele could be contributed by the male), so I denoted both alleles as possibly coming from the father. I assigned an offspring to the last male if all paternal alleles (one or two per locus) at all seven loci were found in the multilocus genotype of the last male. I estimated the minimum number of males contributing to a clutch of a female with the software GERUD 1 (Jones 2001).

Statistical analyses

I performed statistical modelling as recommended in the R Book (Crawley 2007): I started with a maximal model that included all factors, covariates, interactions, and quadratic terms that could be of interest and simplified it in a stepwise manner on the basis of deletion tests (e.g. F tests or chi-squared tests) to the minimal adequate model. Hence, I only included an explanatory variable in a model if it significantly improved the fit of the model (Crawley 2007).
All analyses were performed with R 2.6.2 (R Development Core Team 2008). Linear models were fitted with the *lm* function from the *stats* package, generalized linear models were fitted with the

glm function from the *stats* package, and linear mixed-effects models were fitted with the *lmer* function from the *lme4* package (Bates & Maechler 2008).

I analysed clutch size (i.e. total number of eggs laid) and total number of emerged flies with linear models and square root transformed response. The maximal model included female size, size of the last male, number of yellow dung fly males on cow pat (besides the copulating pair), cow pat age, temperature, and number of alleles or males detected within sperm storage organs as explanatory variables. I analysed the proportion of eggs deposited in the north exposed side of the cow pat and the total proportion of emerged flies with generalized linear models with quasibinomial errors and logit link. I used quasibinomial error structures because models were overdispersed. Explanatory variables were chosen as in the linear models described above. I analysed the minimum number of fathers of a clutch (obtained from the software GERUD 1) with generalized linear models with quasipoisson errors and log link. I used a dispersion parameter since the model was underdispersed. The maximal model included female size, size of the last male, number of alleles or males detected within sperm storage organs, and two-way interactions as explanatory variables. I analysed sperm storage patterns with linear mixed models and log10 transformed number of males detected within each spermatheca as the response. I initially included spermathecal identity, female size, size of the last male, and all two-way interactions as fixed explanatory variables, and female as random effect. I additionally compared the number of different alleles and males (i.e. sires) detected in the offspring to the number of alleles and males detected within the spermathecae with paired t-tests. I also used paired t-tests to compare the number of alleles and males present across the different spermathecae. Residuals in all linear models were normally distributed (all Kolmogorov-Smirnov tests: P = NS). I investigated if females could bias paternity to match the genotypes of their offspring to different environments by comparing last male paternity across the three different environments (N, S, and R). I did this by applying binomial proportions tests *prop.test* from the *stats* package in R. I compared last male paternity in a pairwise fashion (N vs. S, N vs. R, and S vs. R) for every female.

Results

Analyses of clutch size, proportion of eggs laid in the northern exposed side of the cow pat, and sperm storage patterns are based on a sample size of 22 females. In three clutches no flies emerged, presumably because of very wet dung resulting from rainfall that started during oviposition.

Therefore, emergence, paternity and comparison of sperm storage and paternity were analysed with a sample size of 19 females. Statistically significant terms and their p values indicated below are in each case for the minimal adequate model.

Oviposition

Mean clutch size (± SE) was 33.27 ± 2.29 eggs for all 22 females collected in the field, and 34.21 ± 2.59 eggs for the 19 females for which I also had paternity data. I analysed clutch size (i.e. total number of eggs laid) with linear models and square root transformed response. Clutch size significantly increased with female size ($F_{1,18}$ = 14.633, p = 0.001; Fig. 1a). The significant quadratic term for cow pat age ($F_{1,18}$ = 8.353, p = 0.009; Fig. 1b) indicated that clutch sizes were biggest in the middle of the range of time that dung was offered for oviposition. The linear term for cow pat age in the minimal adequate model was not significant ($F_{1,18}$ = 0.239, p = 0.63). Model simplification revealed that the size of the last male, the number of other yellow dung fly males on the cow pat, temperature, and all the interactions included should not be retained in the model as explanatory terms (all p > 0.1).

On average, females laid most of their eggs in the northern exposed side of a cow pat. Mean (± SE) proportions off eggs laid into N, S, and R were: 0.65 ± 0.07, 0.09 ± 0.03, and 0.26 ± 0.06 for all 22 females and 0.65 ± 0.08, 0.10 ± 0.04, and 0.25 ± 0.06 for the 19 females, respectively. I analysed proportion of eggs laid in the northern exposed side of the cow pat with generalized linear models with quasibinomial errors and logit link. The minimal adequate model contained just two parameters: the intercept and temperature. The proportion of eggs laid in the northern exposed side of the cow pat significantly increased with increasing temperature ($F_{1,20}$ = 12.797, p = 0.002; Fig. 2). Model simplification provided no justification for retaining female size, size of the last male, number of other dung fly males on the cow pat, cow pat age, or any interaction in the model (all p > 0.1)

Adult emergence

Mean (± SE) number of emerged flies per clutch was 23.95 ± 2.47 (n = 19 females). I analysed the total number of emerged flies with linear models and square root transformed response. Total number of emerged flies increased significantly with female size ($F_{1,16}$ = 12.220, p = 0.003), and the total number of alleles present in the spermathecae ($F_{1,16}$ = 5.666, p = 0.03; Fig. 3). The size of the

last male and all interactions were not significant and hence omitted during the process of model simplification. Mean (± SE) proportion of emerged flies was 0.69 ± 0.04 (n = 19 females). The proportion of emerged flies was analysed with generalized linear models with quasibinomial errors and logit link. The proportion emerged flies significantly increased with increasing number of alleles detected in the spermathecae ($F_{1,15}$ = 5.143, p = 0.039; Fig. 4). Additionally, the proportion of emerged flies increased with increasing female size only when the female mated last with a big male (significant between female size by last male size interaction: $F_{1,14}$ = 5.514, p = 0.034). Female size ($F_{1,17}$ = 0.101, p = 0.76) and size of the last male ($F_{1,16}$ = 0.018, p = 0.89) in the minimal adequate model did not significantly influence the proportion of emerging flies.

Sperm storage and number of mates

In total I genotyped sperm from 66 spermathecae (22 females x 3 spermathecae). One outer doublet spermatheca provided an unreadable array of alleles and was excluded from analyses. The last male which had mated with the female was always found in all spermathecae. 21 out of 22 females stored sperm from two or more males. On average females stored sperm from 2.82 ± 0.20 males (n = 22 females) or 2.84 ± 0.23 (n = 19 females from whose clutches offspring emerged). I found a significant effect of spermathecal identity on the number of males represented in the sperm store (Markov Chain Monte Carlo p = 0.002, n simulations = 10’000). This significant effect indicated a consistently lower number of ejaculates present in the singlet spermatheca compared to the middle and outer doublet spermatheca (Fig. 5a). Paired t-tests supported this and showed that there was no significant difference in the number of ejaculates between the middle and outer doublet spermathecae: singlet spermatheca vs. middle doublet spermatheca: t = -3.250, df = 21, p = 0.004; singlet spermatheca vs. outer doublet spermatheca: t = -2.905, df = 20, p = 0.009; middle doublet vs. outer doublet spermatheca: t = 0, df = 20, p value = 1. Linear mixed models revealed no significant influence of female size, last male size or any interaction on sperm storage patterns. Mixed model analyses using number of alleles (instead of number of males) as the response variable and paired t-tests based on alleles provided qualitatively the same results as analyses with number of males. The sperm storage patterns based on alleles are shown in Fig 5b.

Paternity

Last male paternity and minimum number of sires for all analysed clutches are given in Table 1. Of the 19 analysed clutches, four clutches only featured eggs laid in the northern exposed side of the

cow pat (i.e. no eggs S and R). This restricted my analyses of differences in last male paternity across N, S, and R to only 15 females. Binomial proportions tests revealed no female that showed differences in last male paternity across N, S, and R (one $p = 0.08$, all other $p > 0.16$). Note that the 15 clutches also include four clutches with complete last male sperm precedence (i.e. all offspring were from the last male). The minimum number of fathers that contribute to a clutch was estimated with the software Gerud and is given in Table 1. I analysed the minimum number of fathers with generalized linear models with quasipoisson errors and log link. The minimum number of fathers estimated for a specific clutch significantly increased with increasing female size ($F_{1,17} = 6.186$, $p = 0.025$), increasing last male size ($F_{1,16} = 7.641$, $p = 0.014$), and the number of males detected within the spermathecae ($F_{1,15} = 19.419$, $p < 0.001$). Generalized linear model analyses with number of alleles detected within spermathecae (instead of number of males) as the explanatory variable (Fig. 6) provided the same results: female size, last male size and number of alleles within spermathecae had a significant positive effect on the number of fathers that contribute to a clutch.

Table 1 Last male paternity and minimum number of sires for 19 wild-caught female yellow dung flies *Scathophaga stercoraria*.

Female	Last male paternity	Minimum number of sires
a	0.881	3
b	0.700	3
c	0.900	3
d	0.682	2
e	0.444	3
f	0.862	2
g	0.886	3
h	0.917	2
I	0.909	2
j	0.800	2
k	0.700	2
l	0.500	2
m	1.000	1
n	0.889	2
o	1.000	1
p	0.917	2
q	1.000	1
r	1.000	1
s	0.850	3
Mean	0.834	2.11

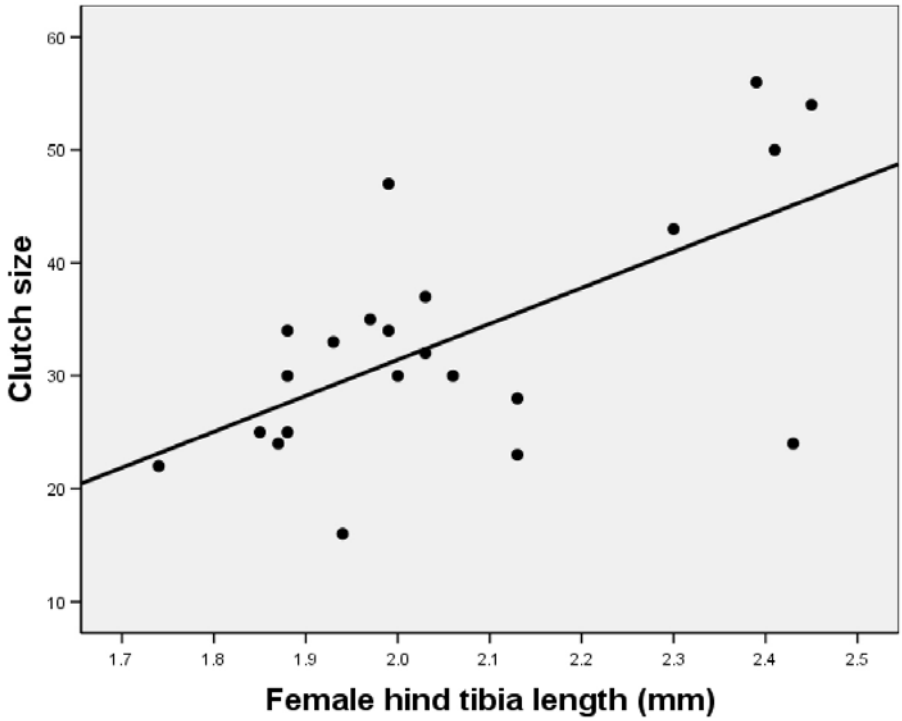

Fig. 1a Clutch size as a function of female size (hind tibia length).

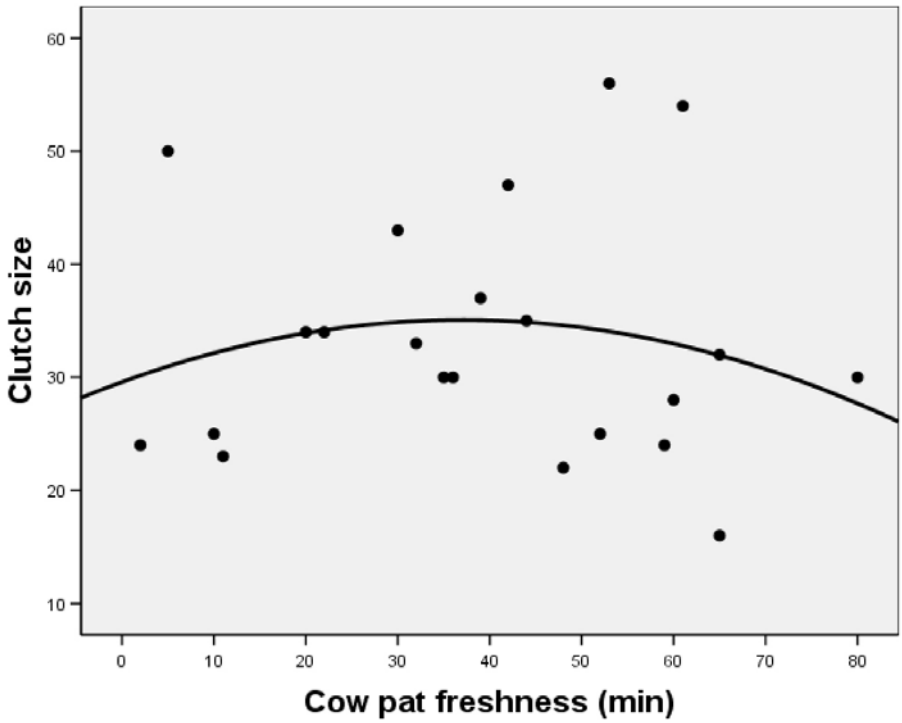

Fig. 1b Clutch size as a function of dung pat age.

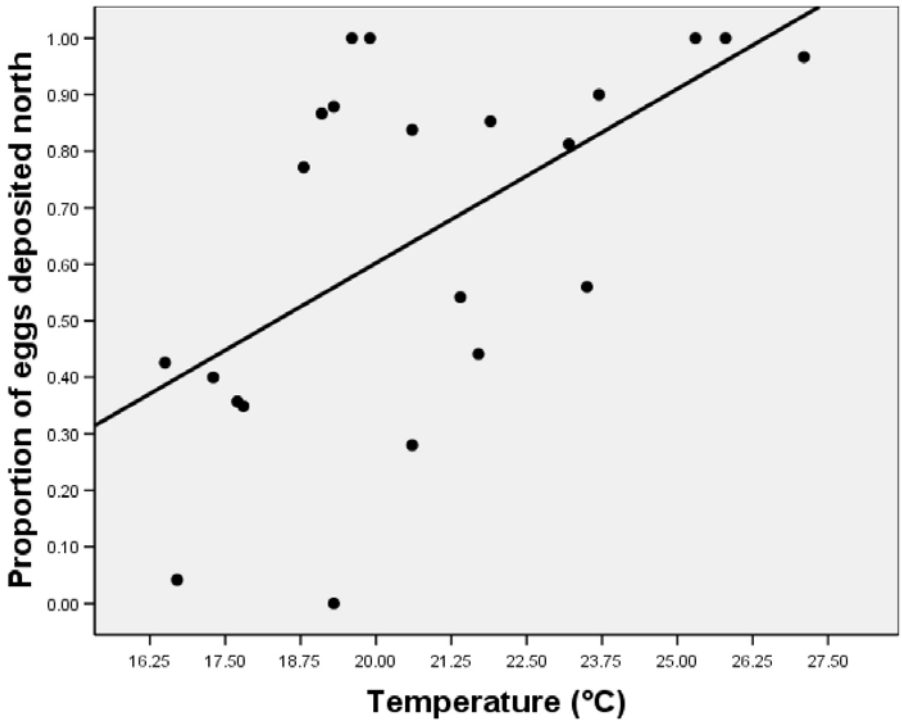

Fig. 2 Proportion of eggs deposited north as a function of temperature.

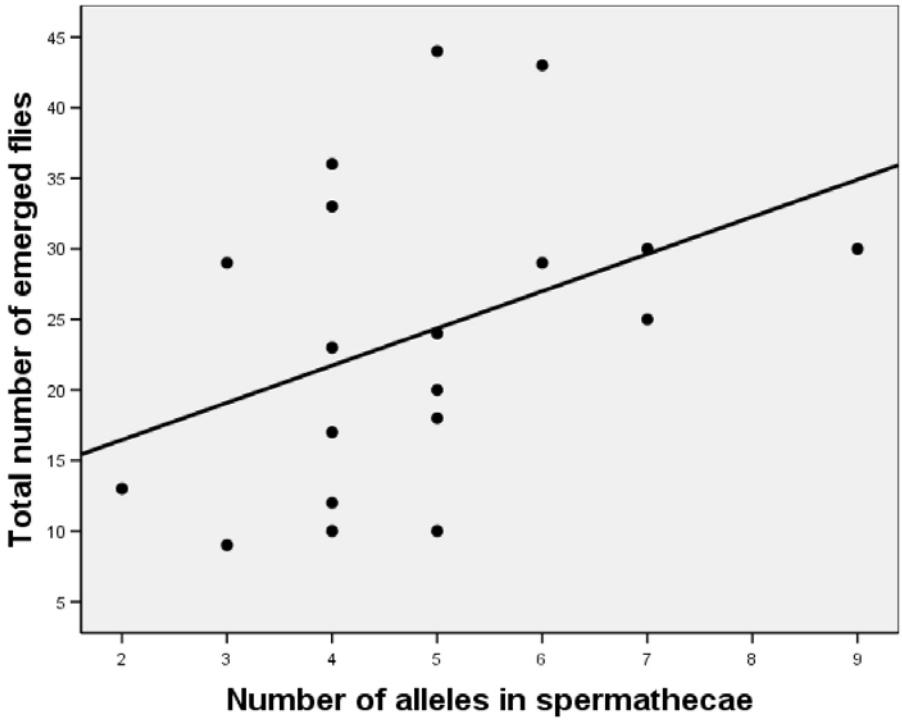

Fig. 3 Total number of emerged flies as a function of the total number of alleles detected within the sperm storage organs (spermathecae) of females.

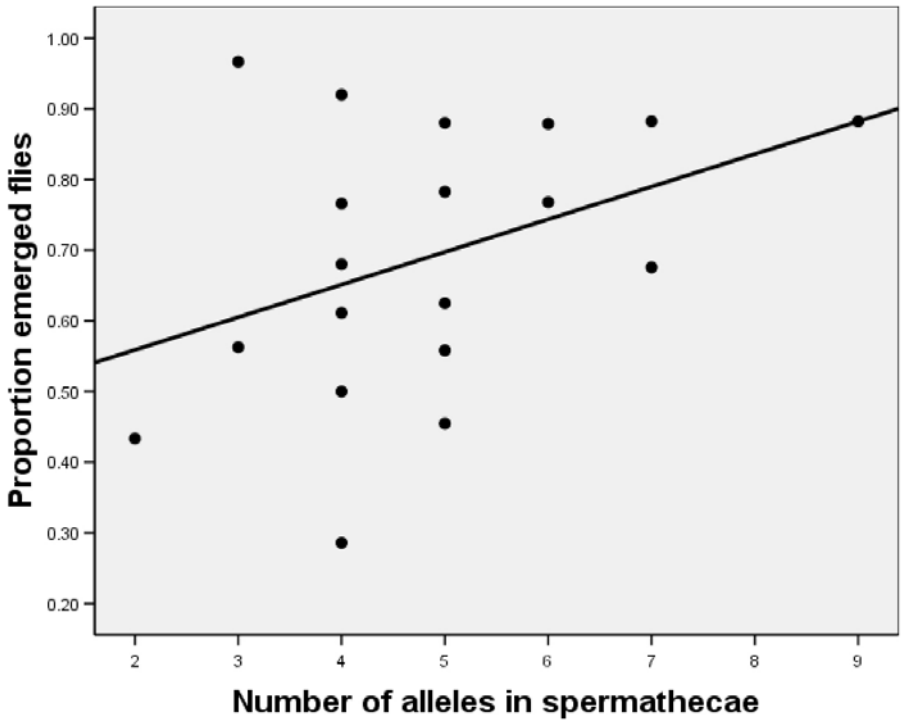

Fig. 4 Proportion of emerged flies as a function of the total number of alleles detected within the sperm storage organs (spermathecae) of females.

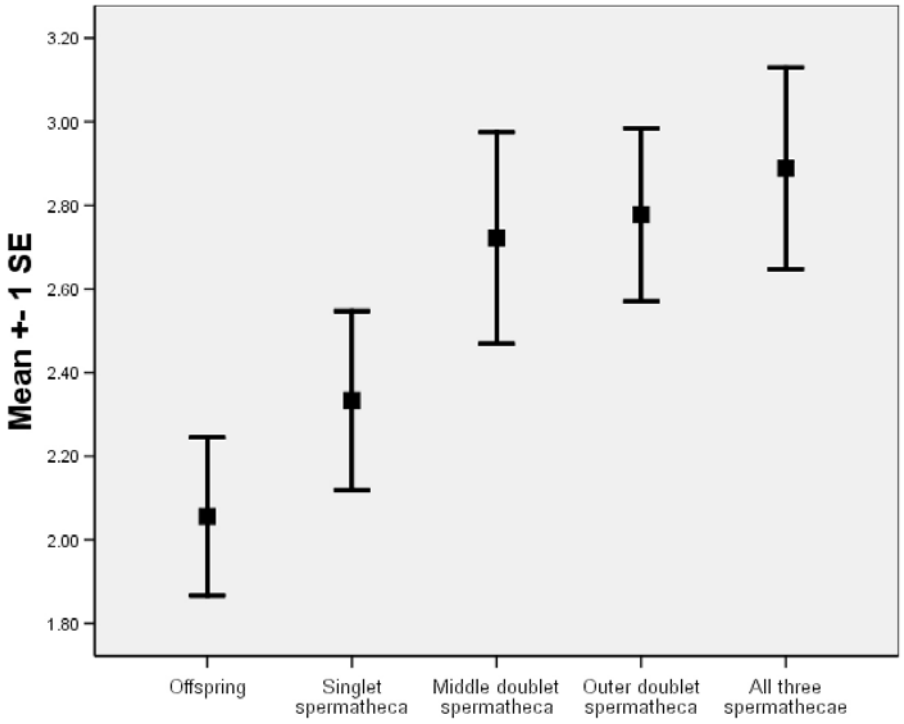

Fig 5a Mean number of males detected within the offspring, particular spermathecae, and all three spermathecae combined.

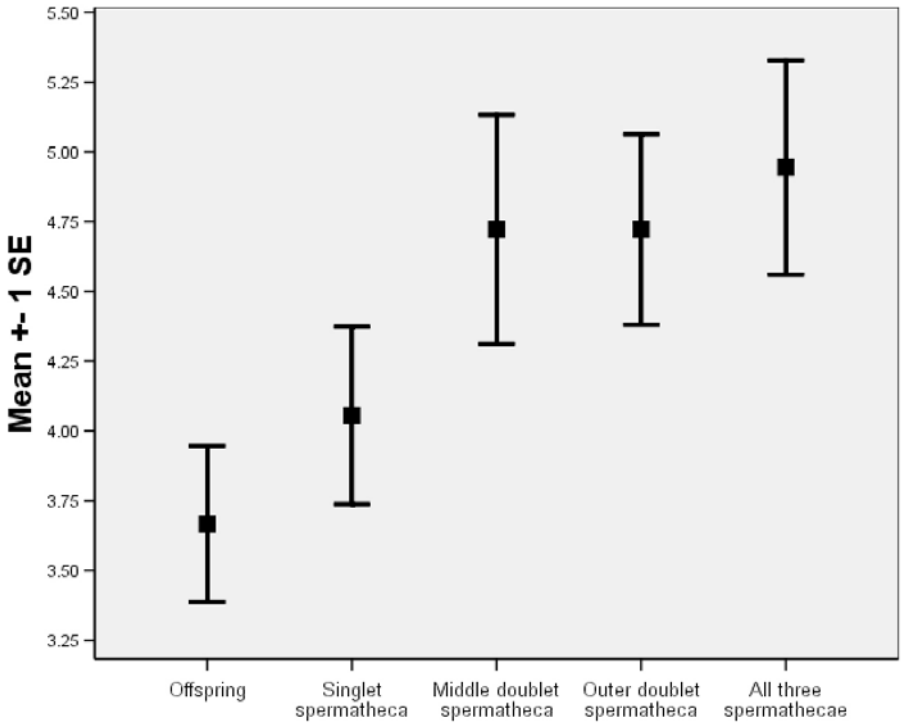

Fig. 5b Mean number of alleles detected within the offspring, particular spermathecae, and all three spermathecae combined.

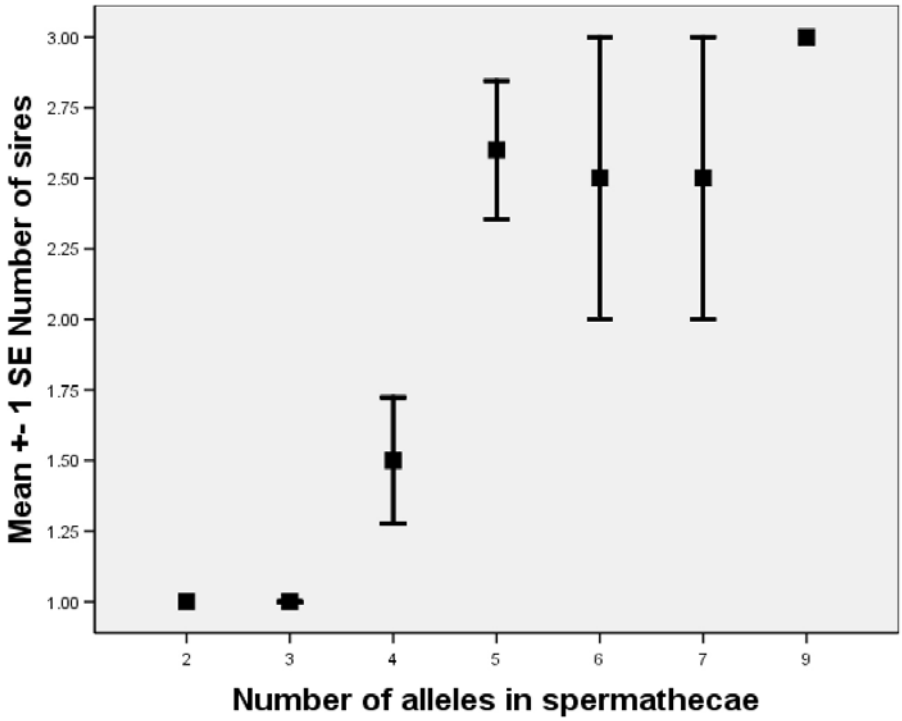

Fig. 6 Mean number of sires for a specific clutch of eggs as a function of the total number of alleles detected within the sperm storage organs (spermathecae) of females.

Discussion

By conducting an oviposition site choice experiment in the field and genotyping the offspring and the sperm from the spermathecae of the females involved, my study provides essential information on sperm storage, paternity, and postcopulatory sexual selection in a natural population of yellow dung flies. On average, females stored sperm from 2.84 males within their sperm stores, indicating high prevailing levels of sperm competition intensity in the field. Interestingly, two independent findings indicate that females may be able to bias paternity toward certain males. First, the number of stored ejaculates differed between the singlet spermatheca and the doublet spermathecae. Second, wild female yellow dung flies stored sperm from more males than sired their offspring. In contrast, my explicit test of sperm selection failed to detect any systematic biasing of paternity of females based on dung pat microclimate (cf. Ward 1998, 2000). Despite the absence of evidence for a sperm selection mechanism, the data show that wild yellow dung fly females can apparently benefit from polyandry via an increased number of emerging offspring.

No evidence of sperm selection based on dung pat microclimate

In the present field study, females could lay their eggs into three distinct areas (environments) on cow pats: the south exposed surface, the north exposed surface, or the ridge in between. Microclimate variation in these cow droppings seems to be substantial (Landin 1967; Ward et al. 2002). Nevertheless, my study revealed no evidence that females match offspring genotypes to prevalent environmental conditions by biasing paternity toward certain fathers from which they have sperm in store. Binomial proportions tests revealed that no single female showed differences in last male paternity across the three experimental cow pat environments. I admit that my sample size was moderate, but the strength of my test of sperm selection based on microclimate was that it did not make any assumptions about a specific trait(s) (e.g. a certain allozyme, any other physiological trait, body size, morphology, etc.) a female would prefer or choose. Obtaining detailed knowledge of the exact male traits females exert preference for is often difficult. Nevertheless, if females are capable of matching offspring genotypes to environmental conditions, they must do so by selecting sperm from certain males at the time of fertilization. The applied binomial proportions tests explicitly tested for this and found no indication of females using sperm differentially in different environments. The last male that had mated with a specific female was always equally successful, irrespective of the environment. Despite the enormous interest in

postcopulatory sexual selection, convincing evidence for sperm selection thus remains very scarce. Previous studies in yellow dung flies provided evidence of sperm selection based on phosphoglucomutase (PGM) alleles (Ward 1998; Ward 2000). However, in these experiments, results were restricted to a small fraction of flies with certain PGM genotypes (the frequency of the most common allele is > 85 % in the field (Ward et al. 2002), strongly constraining the scope for choice), and not all predictions concerning cryptic female choice were confirmed (Ward 1998; Ward 2000). Although female yellow dung flies did not choose what kind of eggs they lay into particular environmental conditions, this does not imply that females are not able to make subtle decisions regarding the placement of eggs or the number of eggs laid. Previous work demonstrated that females prefer to lay their eggs on small hills on the dung surface and avoid depressions and sharply elevated points, featuring decreased reproductive success via increased risks of larvae drowning or drying out respectively (Ward et al. 1999). The present study additionally revealed that temperature and cow pat age strongly influence oviposition. Females laid more eggs at intermediate times after a cow pat had been deposited on a pasture, indicated by the significant quadratic effect of cow pat age on clutch size. The adaptive significance of this behaviour remains to be established. Furthermore, the proportion of eggs deposited into the north exposed surface of a cow pat strongly increased with increasing environmental temperature. Protection of eggs against the negative effects of elevated temperatures and/or desiccation seems the most likely explanation for this behaviour (Ward & Simmons 1990). Thus the present study strengthens the notion that modulation of the number of eggs deposited and the choice of a suitable oviposition sites are much more pronounced in this species than any specific choice regarding paternity of the eggs laid.

Sperm storage, paternity, and the potential for cryptic female choice

Genotyping sperm stores to estimate female mating frequency in natural populations is more useful than genotyping offspring because postcopulatory sexual selection may bias paternity toward certain mates, resulting in an underestimate of existing levels of polyandry in the wild (Bretman & Tregenza 2005; Simmons et al. 2007) (Chapter 4). My study revealed high levels of polyandry (i.e. high sperm competition intensity) in a natural population of yellow dung flies: 21 out of 22 females (95.5 %) stored sperm from two or more males, and on average 2.84 ejaculates compete within the sperm storage organs of females. A related study detected pronounced temporal changes in sperm competition intensity in the same population of yellow dung flies (Chapter 4). Both results from the present study (i.e. the proportion of multiply mated females and the absolute number of competing ejaculates) confirm previous findings for the month of May (Chapter 4).

The sophisticated reproductive tract morphology of yellow dung flies, comprising three spermathecae each with its own duct, may enable females to exert substantial influence over postcopulatory processes (Arthur et al. 2008; Hosken 1999; Hosken et al. 1999). Since males are not able to directly insert sperm into the spermathecae but ejaculate into the bursa copulatrix (Hosken 1999; Hosken et al. 1999; Hosken & Ward 2000; Simmons et al. 1999), sperm transfer to the spermathecae, storage, and displacement are not under direct male control. In particular, theoretical work suggests that females could bias paternity toward certain males by differentially storing sperm from different males in each spermatheca and subsequently choosing sperm (or a sperm mix) from a particular spermatheca (Hellriegel & Ward 1998). These theoretical findings were supported by empirical laboratory studies on yellow dung flies: sperm mixtures indeed differed across the spermathecae in doubly mated females (Bussiere et al. 2009; Hellriegel & Bernasconi 2000). The present study revealed that sperm mixtures also differ in wild yellow dung flies, as I found a significantly lower number of ejaculates present in the singlet spermatheca compared to the middle and outer doublet spermathecae. Again, this result was in accordance with another recent study (Chapter 4) where in contrast to the present study copulations were interrupted. Our two studies, applying slightly different approaches but resulting in the same sperm storage skew across spermathecae, therefore indicate that observed storage patterns in wild female dung flies are robust. Bussière and colleagues (2009) demonstrated that following double matings, the highest proportion of sperm from the second male (S2) was found in the singlet spermatheca. Highest S2 values, and consequently highest displacement of previous sperm in the singlet spermatheca agree with the present field study, as I found the fewest number of ejaculates in the singlet spermatheca. However, it remains to be precisely established why the singlet typically features higher S2 values than either doublet spermatheca (Bussière *et al.* 2009). In particular, it remains unclear whether detecting the fewest males in the singlet spermatheca (in this study) is a result of female influence on sperm storage. Alternatively, this pattern may be due to second (or later) males consistently filling spermathecae in the same order, starting with the singlet.

My study additionally showed that females stored sperm from more males than sired their offspring. The recently developed competitive PCR technique for assessing the proportions of sperm from competing males within females' sperm stores assumes that all genotypes of the males involved are known (Bussière *et al.* 2009). Applying this technique, another study revealed that the amount of stored sperm and achieved paternity success strongly correlate in yellow dung flies following double matings (Chapter 3). In this study I only counted the different ejaculates present across the

spermathecae. Since the genotypes of all involved males (except one) were unknown, I could not quantify the amount of stored sperm for each specific male. Therefore, the present study cannot relate the success or failure of a specific male in obtaining paternity to its amount of stored sperm. Advanced techniques, enabling quantification of the different proportions of stored sperm following multiple matings and/or sperm amounts when the genotypes of involved males are unknown, will be a necessary and fruitful avenue for future research in this field.

As discussed above, I failed to detect sperm selection based on dung pat microclimate. Nevertheless, the complex female reproductive tract morphology (Arthur et al. 2008; Hosken 1999; Hosken et al. 2001; Hosken et al. 1999; Hosken & Ward 2000; Simmons et al. 1999), theoretical considerations (Hellriegel & Ward 1998), and several empirical findings concerning sperm storage and paternity suggest that female yellow dung flies may be able to exert cryptic choice. Sperm contents differed between different spermathecae following double matings in the laboratory (Bussière *et al.* 2009) and multiple matings in the field (this study; Chapter 4). Females stored sperm from more males than sired their offspring (this study), and there is still a considerable amount of unexplained variance regarding paternity success (Simmons & Siva-Jothy 1998). Although it seems that female yellow dung flies have the potential to influence paternity, to date convincing demonstrations of the phenomenon are very limited (Ward 1998; Ward 2000). Future studies should optimize experimental designs making full use of the extensive previous knowledge of this well-studied system. This would allow more careful examination of paternity patterns and the underlying postcopulatory processes (e.g. sperm transfer and storage), potentially clarifying precisely when and to what degree females exert cryptic choice.

Benefits of polyandry

Several laboratory studies have documented benefits of polyandry (Price et al. 2008; Tregenza & Wedell 2002; Zeh & Zeh 2006). In contrast, only few studies have examined polyandry in natural populations and reported benefits (Fisher et al. 2006; Madsen et al. 1992). In yellow dung flies, laboratory studies have shown that multiple mating is associated with longevity costs (Hosken et al. 2002), but that females also benefit from polyandry: more successful males in sperm competition sired offspring that developed faster (Hosken et al. 2003). Here I document benefits of polyandry in a natural population: the proportion and the total number of emerged offspring increased significantly with the number of alleles (my proxy for the number of mating partners) detected within the sperm stores of females. Analyses with the number of males (instead of the number of

alleles) as the explanatory variable provided the same patterns, but were marginally non-significant. The fact that the applied microsatellite markers were highly polymorphic (e.g. all last males involved were at least heterozygous at one locus) suggests that the number of mates *per se* and not heterozygosity is responsible for the observed pattern. Specifically, this implies that the observed pattern of increased fertility with increasing number of alleles does not arise because some females mate with homozygous males and some with heterozygous males. The precise genetic mechanism (e.g. good genes vs. compatible genes) underlying the documented increase in reproductive success of polyandrous females in the field remains to be established.

In summary, my field study showed that female yellow dung flies make subtle decisions regarding the placement of eggs or the number of eggs laid. However, in contrast with some previous studies, there was no evidence of selective use of sperm from particular mating partners according to dung pat microclimate. Nevertheless, the findings that sperm mixtures differed amongst spermathecae and that females stored sperm from more males than sired their offspring clearly undoubtedly indicate potential for cryptic female choice in this species. Future studies have to evaluate when and to what degree these mechanisms can enable females to exert cryptic choice. The present study further revealed intense sperm competition levels in the field and indicated that polyandry has a positive effect on the number of offspring emerging. The precise genetic mechanism underlying the positive effect of multiple mating remains to be established. A better integration of field studies and controlled laboratory experiments is one very promising way to advance our understanding of polyandry and postcopulatory sexual selection processes.

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Summary

Sexual selection arises because individuals vary in reproductive success. It is dominated by two main processes: male competition for access to females (intrasexual selection) and mate choice exerted by choosy females (intersexual selection). Although these categories are most often envisioned to occur before mating, sexual selection is not limited to precopulatory processes, and securing mates is often insufficient for acquiring reproductive success. The two categories of sexual selection also occur after mating, predominantly via two postcopulatory mechanisms: male ejaculates compete for fertilization (sperm competition) and females may exert a preference for the sperm of certain males (cryptic female choice). Some aspects of postcopulatory sexual selection remain controversial, partly because many of these processes are hidden within the bodies of females, and therefore processes determining fertilization success are often inferred from patterns of paternity. As a consequence, mechanisms underlying sperm storage and utilization are largely unknown, and data that directly link the number of stored sperm to paternity are extremely scarce. Hence, the relative contributions of male (sperm competition) and female (cryptic female choice) mechanisms to differential fertilization success, and the extent to which these forces interact, are currently unknown.

Polyandry (the mating of females with more than one male) is a prerequisite for postcopulatory sexual selection. It is a very common phenomenon in insects, but the evolutionary forces favouring multiple mating by females remain very controversial. This is especially true if there are no obvious direct benefits associated with female remating, for example the replenishing of females' sperm stores or the acquisition of nutrients from mating partners. In such cases, repeated mating by females might arise via a number of alternative nonadaptive or adaptive mechanisms, including the acquisition of high quality or compatible genes (indirect benefits). The relative importance of each of these alternatives is currently unknown both in general and for many specific examples of female polyandry. This is partly because research on polyandry has relied heavily on laboratory experiments and it is often unclear whether laboratory findings reflect the conditions experienced by wild populations. Levels of polyandry observed in the lab are particularly suspect because the high densities of lab cultures may encourage more intersexual encounters and harassment than are present in the field. Therefore, to advance our understanding of the evolutionary causes and

consequences of polyandry, we need more information on natural levels of polyandry in wild populations, ideally featuring analyses of its spatial and/or temporal variation.

The yellow dung fly, *Scathophaga stercoraria*, is a model system for studying sexual selection and postcopulatory sexual selection in particular. Even so, field data on sperm storage and paternity are scarce, and the precise mechanisms underlying non-random paternity still unknown. The goal of my PhD thesis was to develop a microsatellite competitive PCR method for quantifying relative contributions of different males to sperm in storage, and apply this method to investigate sperm transfer, storage and utilization (e.g. to establish the relationship between the number of stored sperm and achieved paternity success). The second important purpose was to collect good field data on sperm storage and paternity. Genotyping the sperm stores of females additionally provided a useful estimate of prevailing levels of polyandry in a natural population of dung flies.

In addition to direct studies of the mechanisms involved in sperm transfer, storage and utilization, I was also interested in tests of models of female preference evolution in a quantitative genetic framework, especially insofar as they might shed light on postcopulatory sexual selection mechanisms. **Chapter 1** is an essay about a sexually selected sperm process in the dung beetle *Onthophagus taurus* initially described by Leigh Simmons and Janne Kotiaho. The "sexually selected sperm hypothesis" proposes that postcopulatory sexual selection selects for male traits that increase fertilization efficiency and female traits that promote sperm competition (e.g. multiple mating, complex female reproductive tracts). This hypothesis includes the sexy sperm mechanisms (enhanced fertilization success without enhancement of other fitness-related traits), but does not exclude the possibility that overall genetically superior males have greater fertilization efficiency (the good sperm mechanism). Simmons and Kotiaho applied a quantitative genetic approach to the dung beetle *Onthophagus taurus* to test this idea. They found significant additive genetic variation in spermatheca size, a trait that could play a central role in determining paternity biases. Importantly, consistent with sexy sperm and good sperm processes, their study shows that there is a significant negative genetic correlation between spermatheca size and sperm length: fathers that sired sons with short sperm also sired daughters with large spermathecae.
These results acquire further significance when placed in the context of previous findings in *Onthophagus*. Shorter sperm have a fertilization advantage in competitive situations, and this advantage depends on spermatheca size. Sperm length, like spermatheca size, exhibits

significant additive genetic variance and, interestingly, males in better condition produce shorter sperm. As a result of the genetic covariance between sperm length and male condition, females fertilizing their eggs using shorter sperm could produce offspring of high condition (the good sperm mechanism). Taken together, these findings suggest a sexually selected sperm process incorporating a (good sperm) mechanism to produce high-quality offspring. Postcopulatory sexual selection could thus shape sperm just like precopulatory female preferences affect evolutionary divergence of male secondary sexual traits.
This chapter therefore introduces the compellingly complex interactions underlying postcopulatory sexual selection. The remaining chapters represent empirical efforts to disentangle this complexity using molecular studies of sperm storage and use in the lab and the field.

One methodological challenge in the study of postcopulatory sexual selection is to quantify sperm transfer and storage of individual ejaculates within the reproductive tract of multiply mated females. Previously applied techniques such as radiolabelling and phenotypic markers are practically inferior to genetic markers, because they suffer from potentially confounding influence on sperm movement or completely unambiguous assignment is impossible. **Chapter 2** describes the development and application of microsatellite competitive PCR for quantifying relative contributions to a small number of sperm in storage. We studied how DNA template characteristics affect PCR amplification of known concentrations of mixed DNA, and generated regressions for correcting observations of allelic signal strength based on such characteristics. We used these methods to examine patterns of sperm storage in twice-mated female yellow dung flies, *Scathophaga stercoraria*. We confirmed previous findings supporting sperm displacement and demonstrated that average paternity for the last mate accords with the mean proportion of sperm stored. We further found consistent skew in storage across the three sperm storage organs (spermathecae), with more last male sperm stored in the singlet spermatheca than in either doublet. We also showed that the time between copulations may be important for effectively sorting sperm. Finally, we demonstrated that male size may influence the opportunity for sperm choice, suggesting future work to disentangle the roles of male competition and cryptic female choice.

Using the competitive PCR method developed in the previous chapter, **chapter 3** assessed how biases in sperm storage relate to sperm use during oviposition and female reproductive anatomy. Importantly, by genotyping all offspring from potentially mixed-paternity clutches

we directly estimated the relationship between stored sperm (S2) and paternity success (P2) of the second male. According with the previous chapter, we found consistent skew in sperm storage across the three spermathecae, with more second male sperm stored in the singlet spermatheca than in the doublet. S2 values generally decreased with increasing spermatheca size, possibly indicating less efficient sperm displacement in large spermathecae. Additionally, copula duration and several two-way interactions that included spermathecal identity, female size, and size of the second male significantly influenced S2, highlighting the complexity of postcopulatory processes and sperm storage. Mean S2 for the flies for which we genotyped all offspring was 59.8 % and matched P2 in those flies which was 58.7 %. Importantly, P2 and individual spermathecal S2 values were strongly correlated: 0.902 for the singlet spermatheca; 0.863 for the middle doublet spermatheca; and 0.836 for the outer doublet spermatheca. Oviposition-treatment strongly influenced S2, with S2 being smallest when females laid their eggs directly after the second copula. We argued that the act of laying eggs itself interrupted continued sperm transfer and displacement and caused the smaller S2 values. Interestingly, and contrary to prediction, S2 values were higher when females did not lay eggs than when they oviposited between copulations. Additional analyses across oviposition treatments indicated that reduced copula duration with post-oviposition females (e.g. strategic sperm allocation) explained this pattern. Our study therefore supports the complex network of factors suspected to influence sperm storage. The strong link between the proportion of stored sperm and paternity is most parsimoniously explained by sperm usage that is largely proportional to sperm storage. Nevertheless, substantial unexplained variance and the apparent bias across spermathecae in usage during fertilization could reflect a certain degree of sperm selection by females. Many more data such as these will help clarify the relative contributions of male (sperm competition) and female (cryptic female choice) mechanisms to differential fertilization success.

Polyandry is a prerequisite for postcopulatory sexual selection and very common in insects. Nevertheless, the evolutionary causes and far-reaching consequences of this phenomenon remain debated. **Chapter 4** presents a study of temporal variation in sperm storage and levels of polyandry in a natural population of yellow dung flies. We captured wild female yellow dung flies over the whole spring season and genotyped the sperm from their spermathecae to obtain field information on sperm transfer, storage, and associated levels of polyandry. On average females stored sperm from 2.47 males based on a minimum estimate, and 3.33 based on a probabilistic estimate that incorporates population allele frequencies, respectively. Sperm

storage and therefore sperm competition intensity showed high temporal variation: the proportion of multiply mated females (i.e. females with sperm from ≥ 2 males within their sperm stores) and the absolute number of ejaculates detected within females strongly increased over the spring season before it sharply decreased at the end. Future studies should investigate how males respond to this varying competitive situation. Interestingly, we detected a positive relationship between the number of stored ejaculates and females' wing injuries, suggesting a mechanism by which males may be able to assess prevalent levels of sperm competition intensity. In addition, the number of ejaculates differed amongst the three spermathecae. In agreement with the two previous chapters that documented highest sperm displacement in the singlet spermatheca following double matings in the laboratory, we detected fewer ejaculates in the singlet spermatheca than in either doublet. Currently, we cannot determine whether this skew across spermathecae of wild flies is adaptive, however, some kind of storage bias is a prerequisite for adaptive sperm selection. These field data on sperm transfer and storage provide an important extension to controlled laboratory experiments, and they are essential to validate empirical assessments of the causes and implications of polyandry in laboratory settings.

Chapter 5 was also a field project and built on the previous chapter. I performed an oviposition site choice experiment in a natural population in which female yellow dung flies *S. stercoraria* could deposit their eggs into three different micro-environments on a dung pat (the ridge, north- or south-exposed side), and genotyped the offspring and sperm remaining after oviposition from the three spermathecae of these flies. Temperature strongly influenced egg placement: the warmer the temperature, the higher the proportion of eggs laid into the north-exposed side of dung. The number of ejaculates in storage differed amongst spermathecae as in the previous chapter and females stored sperm from more males than fathered their offspring. Mean last male paternity was 83.4 %, roughly matching some previous estimates from the laboratory, but higher than the reported 58.7 % in chapter 3. Importantly, I found no indication that females are able to lay eggs of different genotypes, by biasing paternity towards certain males, in different places. Thus this represents a strong test of adaptive sperm selection that failed to find any supporting evidence of it. However, this experiment did reveal positive effects of multiple mating on the total number and proportion of offspring emerging from dung. More studies that directly investigate polyandry and cryptic female choice in natural populations will be crucial for critically testing the importance of sperm selection relative to other aspects of postcopulatory sexual selection.

Zusammenfassung

Sexuelle Selektion entsteht weil sich Individuen in ihrem Reproduktionserfolg unterscheiden. Zwei Prozesse dominieren dabei: Männchenkonkurrenz um Zugang zu den Weibchen zu erhalten (intrasexuelle Selektion) und Partnerwahl ausgeübt durch wählerische Weibchen (intersexuelle Selektion). Einen Partner zu ergattern reicht allerdings nicht aus um den Reproduktionserfolg sicherzustellen. Daher finden die zwei Hauptprozesse nicht nur vor der Paarung statt (prekopulatorische sexuelle Selektion), sondern auch während und nach der Paarung (postkopulatorische sexuelle Selektion). Analog zu den zwei Prozessen vor der Paarung, dominieren zwei postkopulatorische Mechanismen: Die Ejakulate der Männchen konkurrieren um die Befruchtung der Eier (Spermienkonkurrenz) und die Weibchen können Spermien gewisser Männchen bevorzugen (kryptische Weibchenwahl). Gewisse Aspekte der postkopulatorischen sexuellen Selektion sind umstritten. Dies liegt zum Teil daran, dass diese Art der Selektion versteckt in den Weibchen drinnen stattfindet, und deswegen die Prozesse, die den Fortpflanzungserfolg bestimmen, häufig nur von der erzielten Vaterschaft abgeleitet werden. Daher sind die Mechanismen, die der Speicherung und Verwendung von Spermien zugrunde liegen weitgehend unbekannt. Des Weiteren sind Daten, welche die gespeicherte Menge der Spermien direkt mit dem erzielten Vaterschaftserfolg in Beziehung setzten, extrem selten. Infolgedessen ist zum jetzigen Zeitpunkt der relative Einfluss der männlichen (Spermienkonkurrenz) und weiblichen (kryptische Weibchenwahl) Mechanismen, sowie deren Interaktion, auf den unterschiedlichen Fortpflanzungserfolg der Individuen, unbekannt.

Polyandrie (die Paarung von Weibchen mit mehr als einem Männchen) ist eine Voraussetzung für die postkopulatorische sexuelle Selektion. Obwohl das Phänomen unter Insekten weit verbreitet ist, sind die evolutionären Kräfte, die mehrfache Paarungen der Weibchen begünstigen, umstritten. Dies trifft vor allem dann zu, wenn Weibchen keinen offensichtlichen direkten Nutzen aus mehrfachen Paarungen ziehen, wie zum Beispiel das Nachfüllen ihres Sperma Vorrates oder den Erhalt von Nahrung vom Paarungspartner. In solchen Fällen kann mehrfaches Verpaaren der Weibchen durch eine Reihe von nicht-adaptiven oder adaptiven Mechanismen, wie zum Beispiel die Akquisition von guten oder kompatiblen Genen (indirekter Nutzen), entstehen. Die relative Bedeutung dieser alternativen Mechanismen für die Entstehung und Erhaltung von Polyandrie im Allgemeinen, aber auch für zahlreiche spezifische Beispiele wo sich Weibchen mehrfach paaren, ist zurzeit unbekannt. Ein wichtiger Grund dafür ist die Tatsache, dass Forschung über Polyandrie vor

allem im Labor stattfindet, und wir deswegen oft nicht wissen, ob unsere experimentellen Designs eine realistische (natürliche) Situation darstellen. Laborresultate können deswegen auch nicht einfach eins zu eins ins Feld übertragen werden. Um unser Verständnis der evolutionären Ursachen und Folgen von Polyandrie zu verbessern, brauchen wir folglich mehr und bessere Daten bezüglich des Levels (Ausmass und Höhe) von Polyandrie in natürlichen Populationen. Idealerweise sollten Studien auch deren räumliche und zeitliche Variation untersuchen.

Die Gelbe Dungfliege, *Scathophaga stercoraria*, ist ein Modellorganismus um die sexuelle Selektion und vor allem die postkopulatorische sexuelle Selektion zu studieren. Nichtsdestotrotz sind Felddaten betreffend Spermien Speicherung und Vaterschaft Mangelware, und die genauen Mechanismen, die der nicht-zufälligen Vaterschaft zugrunde liegen, noch immer unbekannt. Das Ziel meiner Doktorarbeit war es, eine kompetitive Mikrosatelliten PCR zu entwickeln, mit welcher man kleinste Spermien Mengen verschiedener Männchen im Fortpflanzungsapparat der Weibchen quantifizieren kann, und damit die Übertragung, Speicherung und Verwendung von Spermien (z.B. Beziehung zwischen gespeicherter Spermienmenge und erzieltem Vaterschaftserfolg) zu untersuchen. Das zweite wichtige Unterfangen meiner Dissertation bestand darin, wichtige Felddaten bezüglich Spermien Speicherung und Vaterschaft in natürlichen Populationen zu erhalten. Durch das Genotypisieren der Spermien in den Spermien-Speicherorganen der Weibchen (Spermatheken; die Gelbe Dungfliege hat drei: eine einzelne Spermatheke und eine „Doppelspermatheke“) erhält man gleichzeitig auch einen wertvollen Schätzwert über das Level von Polyandrie in natürlichen Populationen von Gelben Dungfliegen.

Zusätzlich zu meinen Projekten, die direkt die involvierten Mechanismen bei der Spermien Übertragung, Speicherung und Verwendung untersucht haben, war ich auch an quantitativen genetischen Studien interessiert, welche Modelle der Evolution von Weibchen-Präferenzen getestet haben. Ich interessiere mich für solche Studien, weil diese ein grosses Potential haben Mechanismen der postkopulatorischen sexuellen Selektion zu beleuchten. **Kapitel 1** ist ein Essay über einen sexuell selektierten Spermien-Prozess im Dungkäfer *Onthophagus taurus*, der ursprünglich von Leigh Simmons und Janne Kotiaho beschrieben wurde. Die „sexuell selektierte Spermien-Hypothese“ schlägt vor, dass die postkopulatorische sexuelle Selektion Männchen Merkmale selektiert (auswählt, bevorzugt), die die Befruchtungs-Effizienz erhöhen, und gleichzeitig aber auch Weibchen Merkmale selektiert, die die

Spermienkonkurrenz fördern (z.B. Mehrfach-Paarungen, komplexe weibliche Fortpflanzungsapparate). Die Hypothese beinhaltet einen „sexy sperm“ Mechanismus (verbesserter Befruchtungserfolg, ohne dass andere Fitnesskomponenten verbessert sind), schliesst aber die Möglichkeit, dass allgemein genetisch bessere Männchen eine grössere Befruchtungs-Effizienz haben („good sperm“ Mechanismus) nicht aus. Simmons und Kotiaho haben diese Hypothese in einer quantitativen genetischen Studie mit *Onthophagus taurus* getestet. Sie entdeckten signifikante additive genetische Varianz für die Grösse der Spermatheken (Spermien-Speicherorgane der Weibchen). Wichtig, passend zu „sexy sperm“ und „good sperm“ Prozessen zeigte die Studie auch eine signifikante negative genetische Korrelation zwischen Spermathekengrösse und Spermienlänge auf: Väter, die Söhne mit kurzen Spermien zeugten, zeugten gleichzeitig auch Töchter mit grossen Spermatheken.
Frühere Studien hatten bereits gezeigt, dass kurze Spermien einen Befruchtungsvorteil besitzen, dass die Spermienlänge signifikante additive genetische Varianz aufweist, und dass Männchen in besserer Kondition kürzere Spermien produzieren. Zusammengefasst legen diese Ergebnisse einen sexuell selektierten Spermien-Prozess nahe, der auch einen „good sperm“ Mechanismus beinhaltet um genetisch hochwertige Nachkommen zu produzieren.
Dieses Kapitel führt in die fesselnde Komplexität postkopulatorischer sexueller Prozesse ein. Die nachfolgenden Kapitel versuchen empirisch, mittels molekularen DNA Methoden, die Komplexität der postkopulatorischen Mechanismen im Labor und Feld zu entschlüsseln.

Eine besonders schwierige methodische Herausforderung beim Studium der postkopulatorischen sexuellen Selektion ist das Quantifizieren der übertragenen und gespeicherten Spermien verschiedener Männchen innerhalb des weiblichen Fortpflanzungsapparates. Bereits angewandte Techniken wie das Markieren durch Radioaktivität oder phänotypische Marker weisen Schwächen auf. **Kapitel 2** beschreibt die Entwicklung und Anwendung einer kompetitiven Mikrosatelliten PCR mit der man kleinste Spermienmengen verschiedener Männchen in den Spermien-Speicherorganen der Weibchen quantifizieren kann. Wir studierten wie die Eigenschaften der DNA Matrize (template) die PCR Amplifikation von bekannten Konzentrationen von DNA Gemischen beeinflusst, und generierten Regressionen um die Signalstärke der Allele nach der PCR zu korrigieren. Wir verwendeten die Methode um die Spermien Speicherung bei zweifach verpaarten Weibchen der Gelben Dungfliege zu untersuchen. Wir bestätigten frühere Resultate, welche besagen, dass Spermien Verdrängung stattfindet und dass der durchschnittliche Vaterschaftserfolg dem durchschnittlichen Anteil der gespeicherten Spermienmenge entspricht. Des Weiteren

entdeckten wir konsistente Unterschiede in der Spermien Speicherung zwischen den drei Spermatheken: Es befand sich mehr Sperma des letzten Männchens in der einzelnen Spermatheke als in der mittleren oder äusseren „Doppelspermatheke“. Wir zeigten auch, dass die Zeit zwischen zwei Paarungen entscheidend sein kann, um die Spermien zweier Männchen effektiv zu trennen. Schlussendlich zeigte das Projekt auch, dass die Männchengrösse die Fähigkeit der Weibchen zur Spermienwahl beeinflussen könnte.

Kapitel 3 verwendet die im vorherigen Kapitel entwickelte kompetitive Mikrosatelliten PCR und untersucht damit die Beziehung zwischen Spermien Speicherung und Verwendung während der Eiablage und zwischen Spermien Speicherung und den Dimensionen des weiblichen Fortpflanzungsapparates. Wichtig, indem wir auch alle Nachkommen genotypisieren, die potenziell von verschiedenen Vätern abstammen, können wir auch die gespeicherte Spermienmenge vom zweiten Männchen (S2) zu dessen erzieltem Vaterschaftserfolg (P2) in Beziehung setzen. In Übereinstimmung mit dem vorherigen Kapitel fanden wir konsistente Unterschiede in der Spermien Speicherung zwischen den Spermatheken, wobei mehr Spermien vom zweiten Männchen in der einzelnen Spermatheke gespeichert waren als in der „Doppelspermatheke“. Je grösser die Spermatheken waren, umso kleiner waren die S2 Werte. Dies ist ein Hinweis darauf, dass die Spermien Verdrängung in grossen Spermatheken weniger effizient ist. Des Weiteren beeinflusste die Kopulationsdauer und mehrere Zwei-Weg Interaktionen, welche die Spermatheke, die Weibchengrösse und die Grösse des zweiten Männchens beinhalteten, die S2 Werte. Diese vielfältigen signifikanten Einflüsse unterstreichen die Komplexität der postkopulatorischen Prozesse und der Spermien Speicherung. Bei den Fliegen, von denen wir auch alle Nachkommen genotypisiert hatten, entsprach der S2 Mittelwert (59.8 %) dem P2 Mittelwert (58.7 %). Wichtig, die einzelnen S2 und P2 Werte korrelierten ebenfalls stark: 0.902 die einzelne Spermatheke; 0.863 die mittlere „Doppelspermatheke“; und 0.836 die äussere „Doppelspermatheke“. Das Eiablage-Treatment hatte einen starken Einfluss auf S2, wobei S2 am kleinsten war wenn die Eiablage direkt nach der zweiten Kopulation stattfand. Wir erklärten diesen Befund damit, dass die Eiablage den kontinuierlichen Spermien Transfer zur Spermatheke und die resultierende Verdrängung bereits gespeicherter Spermien unterbrochen hat und damit die niedrigeren S2 Werte verursacht hat. Erstaunlicherweise waren die S2 Werte grösser wenn keine Eiablage stattgefunden hat, als wenn die Weibchen zwischen der ersten und zweiten Kopulation Eier legen konnten. Zusätzliche Analysen konnten aufzeigen, dass verkürzte zweite Kopulationen

mit den Weibchen die vorher gerade Eier gelegt haben (strategisches Ejakulieren) für diesen Befund verantwortlich waren.
Die starke Beziehung zwischen S2 und P2 deutet an, dass die Spermien Verwendung in Gelben Dungliegen weitgehend proportional zur gespeicherten Spermienmenge ist (d.h. zufällig ist). Substantielle nicht-erklärte Varianz in der Beziehung zwischen S2 und P2 Werten und unterschiedlich starke Korrelationen zwischen S2 und P2 für die drei Spermatheken deuten dennoch an, dass ein gewisses Mass der Spermienselektion durch Weibchen durchaus möglich ist. Mehr Daten wie die, die in diesem Kapitel präsentiert wurden, werden helfen die relativen Einflüsse der Männchen (Spermienkonkurrenz) und Weibchen (kryptische Weibchenwahl) auf den unterschiedlichen Befruchtungserfolg der Individuen zu klären.

Polyandrie ist eine Voraussetzung für die postkopulatorische sexuelle Selektion. Sie kommt bei Insekten extrem häufig vor. Nichtsdestotrotz, die evolutionären Ursachen und weitreichenden Folgen dieses Phänomens sind umstritten. **Kapitel 4** präsentiert eine Studie zur zeitlichen Variation in der Spermien Speicherung und zum Level von Polyandrie in einer natürlichen Population von Gelben Dungfliegen. Wir sammelten wilde Dungfliegen Weibchen während des ganzen Frühlings und genotypisierten die Spermien, die in den Spermatheken gespeichert waren. Wir erhielten dadurch Felddaten über die Spermien Übertragung, Speicherung und das damit verknüpfte Level der Polyandrie. Im Schnitt speicherten die Weibchen Spermien von 2.47 (minimaler Schätzwert) beziehungsweise 3.33 (wahrscheinlichkeitstheoretischer Schätzwert der die Populationsallelfrequenzen miteinbezieht) Männchen. Spermien Speicherung und damit auch die Intensität der Spermienkonkurrenz wiesen eine ausgeprägte zeitliche Variation auf: der Anteil der Weibchen die mehrfach verpaart waren und die absolute Anzahl der Ejakulate in den Weibchen stieg über den ganzen Frühling stark an, bevor sie ganz am Schluss (zweite Juni Hälfte) stark zusammenbrach. Zukünftige Studien sollten untersuchen, wie die Männchen auf diese variierende Intensität der Spermienkonkurrenz reagieren. Interessanterweise entdeckten wir eine positive Beziehung zwischen der Anzahl der gespeicherten Ejakulate und der Anzahl der grossen Flügelverletzungen bei Weibchen. Grosse Flügelverletzungen könnten daher den Männchen einen einfachen Anhaltspunkt über die vorherrschende Intensität der Spermienkonkurrenz liefern. Des Weiteren unterschied sich die Anzahl der Ejakulate in den drei Spermatheken. Am wenigsten Ejakulate waren in der einzelnen Spermatheke gespeichert. Dieses Resultat stimmt mit den vorherigen zwei Kapiteln überein, die aufgezeigt hatten, dass

die Spermien Verdrängung in der einzelnen Spermatheke am stärksten ausgeprägt ist. Es bleibt abzuklären, inwiefern diese Unterschiede zwischen den Spermatheken von wilden Dungfliegen Weibchen adaptiv sind. Die Unterschiede bezüglich Inhalt der Spermien zwischen den drei Spermatheken scheinen aber auf jeden Fall eine Voraussetzung für Spermienselektion durch Weibchen zu sein. Felddaten bezüglich der Spermien Speicherung und dem Level von Polyandrie sind eine notwendige Ergänzung zu Laborexperimenten. Daten aus natürlichen Populationen helfen auch Laborexperimente zu validieren und zu verbessern.

Kapitel 5 ist ebenfalls ein Feldprojekt. Ich führte ein Eiablage Experiment durch, bei dem Gelbe Dungfliegen Weibchen wählen konnten in welcher von drei Mikro-Umwelten (nördlicher oder südlicher Hang eines künstlichen Kuhfladens, bzw. Kante die von den zwei Hängen gebildet wurde) sie ihre Eier legen. Ich genotypisierte alle Nachkommen und das Sperma in den Spermatheken. Die Temperatur hatte einen starken Einfluss auf die Eiablage: je wärmer es war, desto mehr Eier legten die Weibchen in den nördlichen Hang. Wie im vorherigen Kapitel unterschieden sich die Spermatheken in der Anzahl der gespeicherten Ejakulate, und nicht alle Männchen die in den Spermatheken vertreten waren zeugten Nachkommen. Die durchschnittliche Vaterschaft des letzten Männchens war 83.4 %. Diese Zahl entspricht einigen früheren Studien, ist aber höher als die im Kapitel 3 (58.7%). Wichtig, ich fand absolut keinen Hinweis darauf, dass die Weibchen in der Lage sind die Vaterschaft den vorherrschenden Umweltbedingungen anzupassen: der Vaterschaftserfolg eines jeweiligen letzten Männchens war in allen drei Mikro-Umwelten gleich gross. Meine Studie fand daher keinen Hinweis auf adaptive Spermienselektion durch Weibchen. Dafür deckte meine Studie einen positiven Einfluss von Polyandrie auf die Anzahl von geschlüpften Nachkommen auf. Weitere Feldprojekte die Polyandrie und kryptische Weibchenwahl direkt in natürlichen Populationen untersuchen sind unverzichtbar, um zu verstehen wie wichtig Spermienselektion durch Weibchen im Vergleich zu anderen postkopulatorischen Prozessen ist.

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Curriculum Vitae

Name	Demont
Vorname	Marco
Geburtsdatum	5. März 1977 in Chur, Schweiz
Heimatort und Kanton	Vella, Graubünden
Nationalität	Schweizer

Ausbildung

1992 – 1997	Bündner Kantonsschule Chur
1997	Matura Typus C
1997 – 1998	Medizinstudium an der Universität Basel
1998	Erstes Vordiplom für Ärzte, Zahnärzte und Tierärzte
1998 – 2000	Grundstudium der Biologie an der Universität Basel
1999	Erstes Vordiplom in Biologie
2000	Zweites Vordiplom in Biologie
2001 – 2004	Hauptstudium der Biologie an der Universität Zürich
2003 – 2004	Diplomarbeit am Zoologischen Museum der Universität Zürich
	Titel der Diplomarbeit: „Latitudinal Clinal Differentiation of Yellow Dung Fly Life History Traits across Europe: A Comparison of Molecular and Quantitative Genetic Data”
2004	Diplom in Zoologie
2005	Wissenschaftlicher Mitarbeiter am Zoologischen Museum der Universität Zürich
	Unterrichtspraktikum an der Alten Kantonsschule Aarau
2006 – 2010	Dissertation am Zoologischen Museum der Universität Zürich unter der Leitung von Dr. Luc F. Bussière und Prof. Dr. Wolf U. Blanckenhorn
	Titel der Dissertation: „Polyandry and Postcopulatory Sexual Selection in Yellow Dung Flies“